A tumour suppressor role for FOXP3 and FOXP3-regulated microRNAs in breast cancer cells

NATASHA JACQUELINE MCINNES

Discipline of Paediatrics, School of Paediatrics & Reproductive Health, University of Adelaide

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Thesis Corrigendum

"A tumour suppressor role for FOXP3 and FOXP3-regulated microRNAs in breast cancer cells"

Natasha Jacqueline McInnes

September 2012

A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy

This corrigendum has been included in this thesis due to errors identified in the analysis of Quantitative Real-Time PCR experiments that were performed after this thesis was submitted. These errors were typographical, and arose in the manual transfer of numbers from raw data files (Rotorgene 6000 software) to data analysis files (Microsoft Excel software). It is therefore possible that errors of a similar nature arose during the preparation of figures for this thesis.

Importantly, it should be noted that despite the potential minor errors present in the Quantitative Real-Time PCR data presented in this thesis, this was not the only method used to investigate the hypotheses. Additional experiments that support the results of the Quantitative Real-Time PCR experiments include western blots, luciferase assays and growth assays. It is therefore extremely unlikely that the presence of minor errors in the Quantitative Real-Time PCR analyses would influence the overall significance and conclusions of this thesis.

Unfortunately, due to misplacement of the data files used to generate the figures in this thesis, it is not possible to provide replacement figures for the Quantitative Real-Time PCR experiments performed. However, one experiment relating to the work performed in Chapter 4 of this thesis was performed after thesis submission, and supports Figure 4.6a of this thesis. The results of this additional experiment and a comparison with the results shown in Figure 4.6a are discussed in further detail on this disc.

Natasha Jacqueline McInnes

Date: 3/11/14

corbett RESEARCH
A Division of Corbett Life Science

14 Hilly Street Mortlake NSW 2137 Australia
T +61 2 9736 1320
F +61 2 9736 1364
W www.corbettlifescience.com

Quantitation Report

Experiment Information

Run Name	RPL13a SATB1 1.3.13
Run Start	1/03/2013 11:56:39 AM
Run Finish	1/03/2013 12:58:59 PM
Operator	natasha
Notes	BT 231 PNA and pre-miR 155: SATB1 and RPL13a
Run On Software Version	Rotor-Gene 4.4.1
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

Threshold	0.01832
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

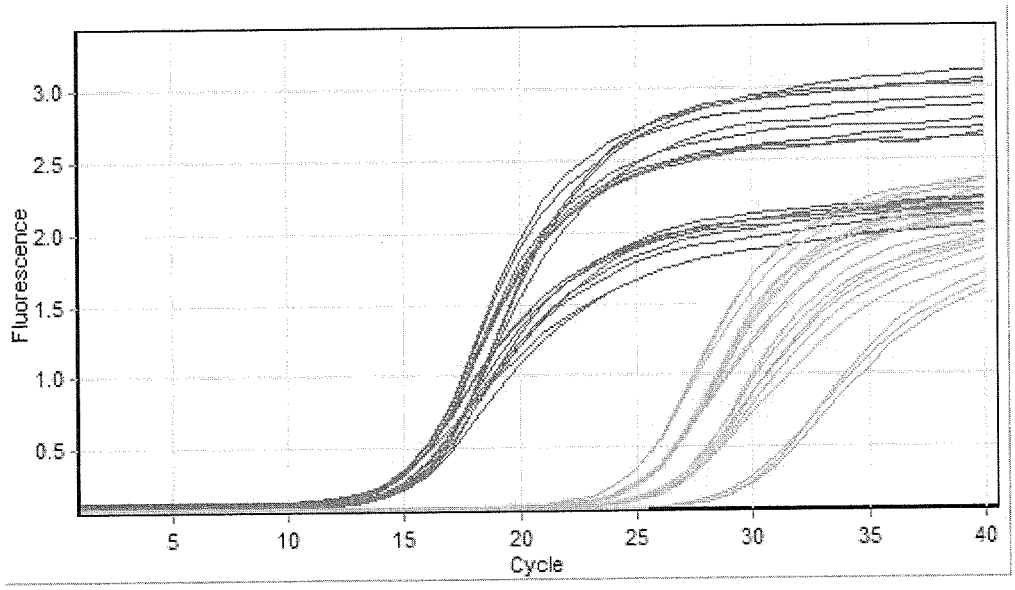
Messages

Message

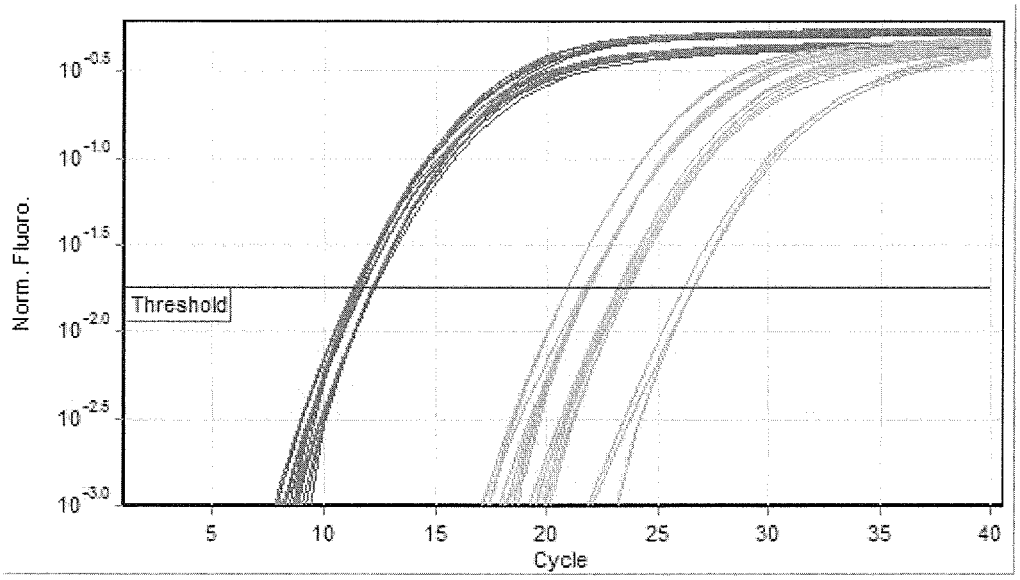
Profile

Cycle	Cycle Point
Hold @ 95°c, 3 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 3 secs
	Step 2 @ 60°c, hold 25 secs, acquiring to Cycling A([Green][1][1])
Melt (50-99°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

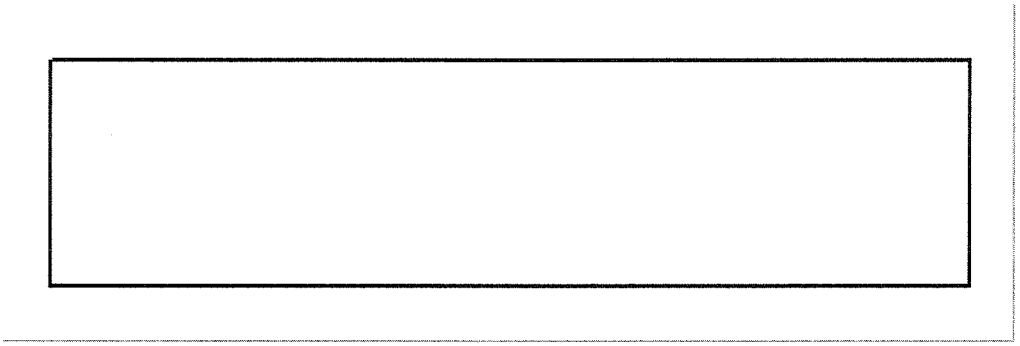
Raw Data For Cycling A.Green



Quantitation data for Cycling A.Green



Standard Curve



No.	Colour	Name	Type	Ct	Rep. Ct	Rep. Ct Std. Dev.	Rep. Ct (95% CI)
34		Bt alone RPL	Unknown	12.24	12.21	0.02	[12.16 , 12.27]
35		Bt alone RPL	Unknown	12.19			
36		Bt alone RPL	Unknown	12.22			
37		Bt+miR NC RPL	Unknown	11.51	11.52	0.08	[11.32 , 11.72]
38		Bt+miR NC RPL	Unknown	11.45			
39		Bt+miR NC RPL	Unknown	11.60			
40		Bt+miR-155 RPL	Unknown	11.58	11.57	0.03	[11.50 , 11.63]
41		Bt+miR-155 RPL	Unknown	11.58			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Rep. Ct	Rep. Ct Std. Dev.	Rep. Ct (95% CI)
42		Bt+miR-155 RPL	Unknown	11.54			
49		231 alone RPL	Unknown	11.79	11.75	0.05	[11.64 , 11.86]
50		231 alone RPL	Unknown	11.76			
51		231 alone RPL	Unknown	11.70			

52		231+miR NC RPL	Unknown	11.36	11.37	0.01	[11.35 , 11.39]
53		231+miR NC RPL	Unknown	11.37			
54		231+miR NC RPL	Unknown	11.37			
55		231+miR-155 RPL	Unknown	12.23	12.25	0.07	[12.09 , 12.42]
56		231+miR-155 RPL	Unknown	12.33			
57		231+miR-155 RPL	Unknown	12.20			
64		Bt alone SAT	Unknown	21.73	21.82	0.09	[21.60 , 22.05]
65		Bt alone SAT	Unknown	21.92			
66		Bt alone SAT	Unknown	21.82			
67		Bt+miR NC SAT	Unknown	20.97	20.96	0.00	[20.95 , 20.97]
68		Bt+miR NC SAT	Unknown	20.96			
69		Bt+miR NC SAT	Unknown	20.96			
70		Bt+miR-155 SAT	Unknown	21.82	21.84	0.04	[21.74 , 21.95]
71		Bt+miR-155 SAT	Unknown	21.89			
72		Bt+miR-155 SAT	Unknown	21.82			
79		231 alone SAT	Unknown	23.51	23.60	0.08	[23.40 , 23.80]
80		231 alone SAT	Unknown	23.64			
81		231 alone SAT	Unknown	23.66			
82		231+miR NC SAT	Unknown	23.16	23.26	0.11	[22.98 , 23.53]
83		231+miR NC SAT	Unknown	23.24			
84		231+miR NC SAT	Unknown	23.38			
85		231+miR-155 SAT	Unknown	26.60	26.45	0.26	[25.81 , 27.08]
86		231+miR-155 SAT	Unknown	26.59			
87		231+miR-155 SAT	Unknown	26.15			

Warning: The following samples were not analysed :
43-Bt+PNA NC RPL 44-Bt+PNA NC RPL 45-Bt+PNA NC RPL 46-Bt+PNA-155 RPL 47-Bt+PNA-155 RPL 48-Bt+PNA-155 RPL 58-231+PNA NC RPL 59-231+PNA NC RPL 60-231+PNA NC RPL 61-231+PNA-155 RPL 62-231+PNA-155 RPL 63-231+PNA-155 RPL 73-Bt+PNA NC SAT 74-Bt+PNA NC SAT 75-Bt+PNA NC SAT 76-Bt+PNA-155 SAT 77-Bt+PNA-155 SAT 78-Bt+PNA-155 SAT 88-231+PNA NC SAT 89-231+PNA NC SAT 90-231+PNA NC SAT 91-231+PNA-155 SAT 92-231+PNA-155 SAT 93-231+PNA-155 SAT

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

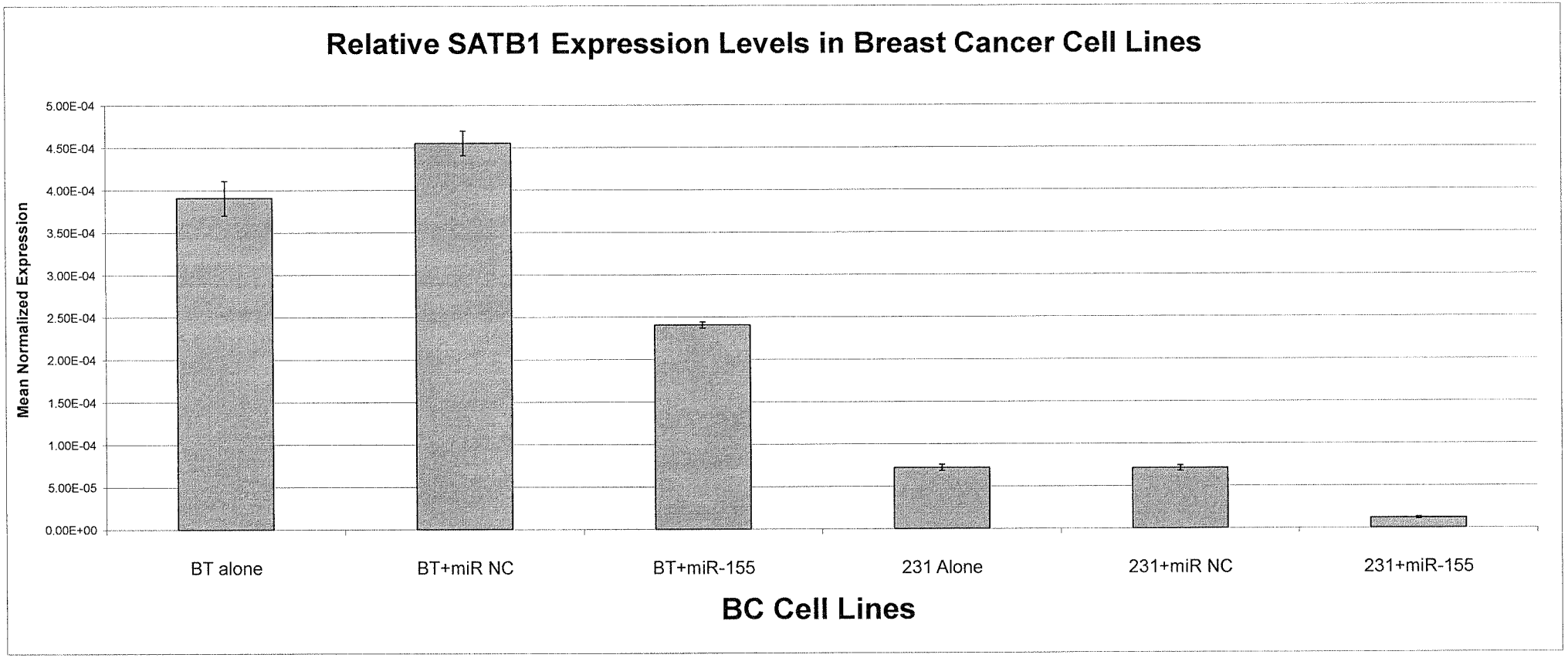
SATB1 (BT549 & MDA-MB-231)

Calculation Procedure for MNE	1
Threshold for SEM in %	20.0

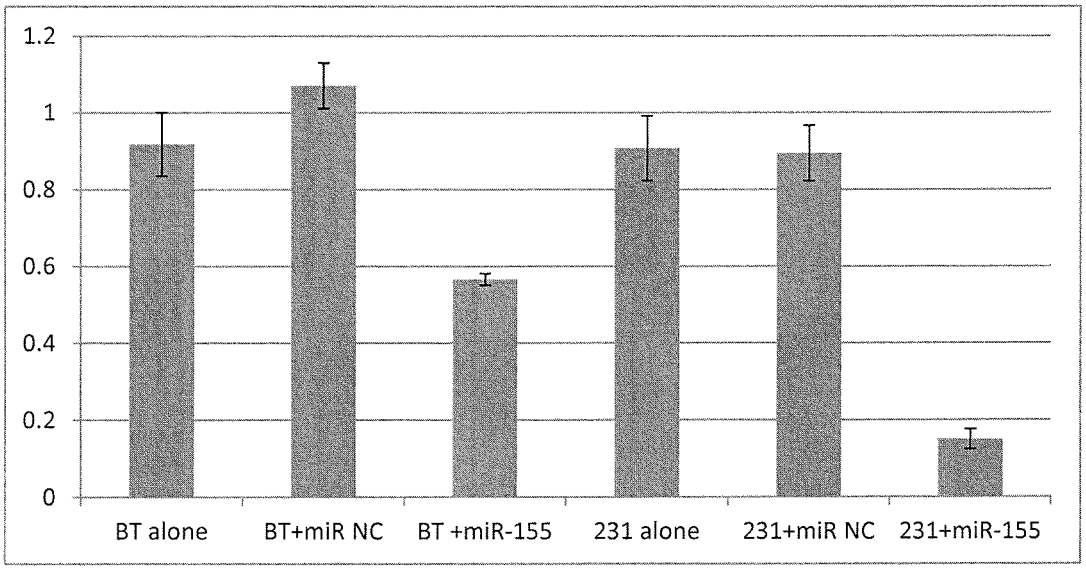
Well	Description	CT of Target Gene	CT of Reference Gene	Normalized Expression
A1	BT alone	21.73	12.24	4.25E-04
A2		21.92	12.19	3.55E-04
A3		21.82	12.22	3.92E-04
A4	BT+miR NC	20.97	11.51	4.49E-04
A5		20.96	11.45	4.34E-04
A6		20.96	11.60	4.83E-04
A7	BT+miR-155	21.82	11.58	2.47E-04
A8		21.89	11.58	2.35E-04
A9		21.82	11.54	2.40E-04
A10	231 Alone	23.51	11.79	7.94E-05
A11		23.64	11.76	7.04E-05
A12		23.66	11.70	6.64E-05
B1		23.16	11.36	7.62E-05
B2	231+miR NC	23.24	11.37	7.22E-05
B3		23.38	11.37	6.49E-05
B4		26.60	12.23	1.04E-05
B5	231+miR-155	26.59	12.33	1.12E-05
B6		26.15	12.20	1.43E-05
B7				
B8				
B9				
B10				
B11				
B12				
C1				
C2				
C3				
C4				
C5				
C6				
C7				
C8				
C9				
C10				
C11				
C12				
D1				
D2				
D3				
D4				
D5				
D6				
D7				
D8				
D9				
D10				
D11				

Data Analysis

Mean of Triplicates			
Description	Mean Normalized Expression	SE of Mean Normalized Expression	SE of Mean Normalized Expression in %
BT alone	3.91E-04	2.03E-05	5.19
BT+miR NC	4.55E-04	1.45E-05	3.19
BT+miR-155	2.41E-04	3.71E-06	1.54
231 Alone	7.21E-05	3.85E-06	5.34
231+miR NC	7.11E-05	3.30E-06	4.65
231+miR-155	1.20E-05	1.19E-06	9.96

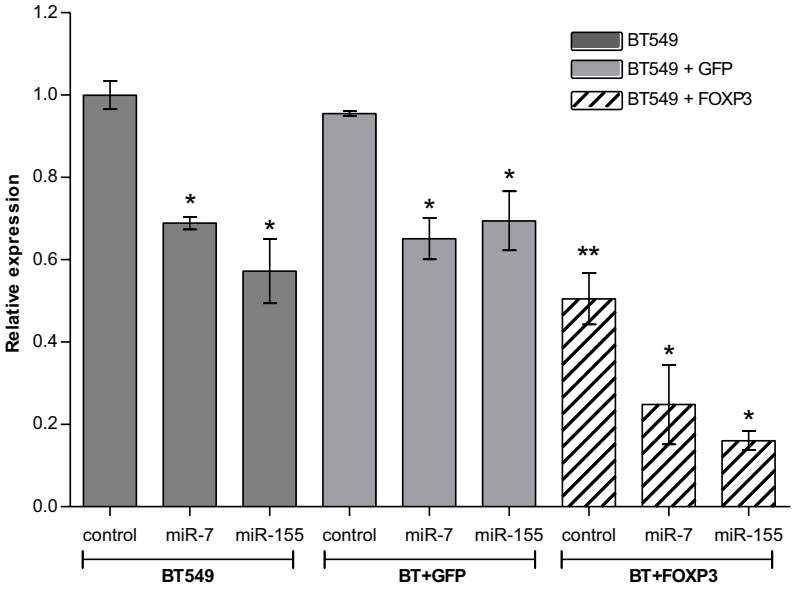

Relative SATB1 Expression Levels in Breast Cancer Cell Lines
Mean Normalized Expression
5.00E-04
4.50E-04
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
BT alone
BT+miR NC
BT+miR-155
231 Alone
231+miR NC
231+miR-155
BC Cell Lines

Cell line/treatment	Normalised expression	Relative expression	Average	Standard Deviation
	0.000425385	1		
	0.000355122	0.834825725		
BT alone	0.000391554	0.920468984	0.918432	0.082605984
	0.000449382	1.056413368		
	0.000433737	1.019635089		
BT+miR NC	0.000483021	1.135491629	1.070513	0.059201282
	0.000247384	0.581552229		
	0.000234544	0.55136967		
BT+miR-155	0.000240385	0.565099532	0.566007	0.015111735
	7.94326E-05	1		
	7.04151E-05	0.886476064		
231 alone	6.64289E-05	0.836292587	0.90759	0.083871114
	7.61627E-05	0.958834507		
	7.21782E-05	0.908672108		
231+miR NC	6.48805E-05	0.816799557	0.894769	0.072030964
	1.03604E-05	0.130430006		
	1.12161E-05	0.141202864		
231+miR-155	1.42831E-05	0.17981439	0.150482	0.025967036

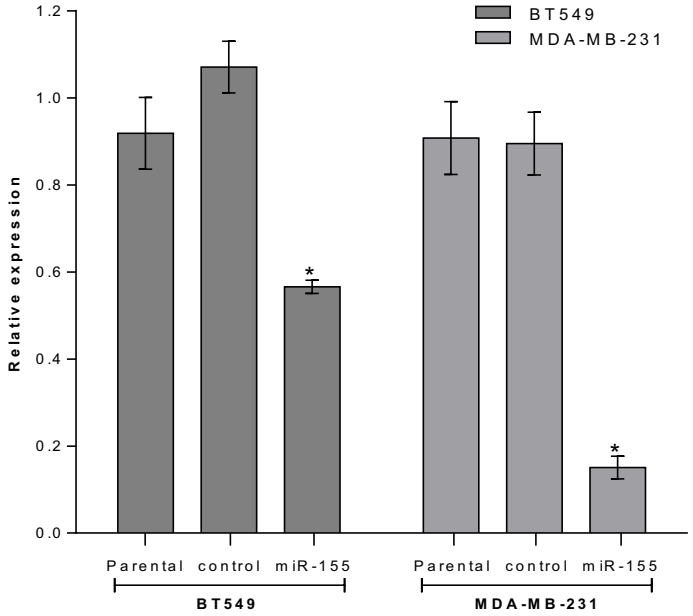


Corrigendum Figure 1 ***Endogenous SATB1 expression is reduced by microRNAs***

a)



b)



a) Figure 4.6a as it appears in this thesis. Expression of endogenous *SATB1* mRNA is reduced when pre-miR-7 or pre-miR-155 is transiently expressed in BT549 cells. *SATB1* levels in the parental cells (dark bars) and GFP-transduced control lines (grey bars) are reduced by the transfection of miR-7 or miR-155. Overexpression of *FOXP3* alone (hatched bars) reduces *SATB1* levels, compared with the control cell lines. Transient transfection of either pre-miR further reduces *SATB1* in these cells. (Triplicate RNA analysis of n=3 transfection pools, *$p<3.12x10^{-5}$, **$p=1.03x10^{-12}$. b) Figure derived from an experiment performed after this thesis was submitted. Expression of endogenous *SATB1* mRNA is reduced when pre-miR-155 is transiently expressed in BT549 (dark bars) and MDA-MB-231 (grey bars) breast cancer cell lines. Transient transfection of parental cells with a pre-miR control does not reduce *SATB1* levels, while transient transfection with pre-miR-155 result in significantly reduced *SATB1* levels. (Triplicate RNA analysis of n=1 transfection pool, *$p<0.0002$).

Corrigendum Discussion

In this thesis, the quantitative RT-PCR analysis shown in Figure 4.6a (and in Corrigendum Figure 1a) demonstrates that transient transfection of microRNA precursors pre-miR-7 and pre-miR-155 into the BT549 cell lines resulted in a significant reduction in the *SATB1* mRNA levels when compared with the control pre-miR-transfected lines (35 to 38% reduction, $p=6.35x10^{-5}$, and 35 to 45% reduction, $p=1.4x10^{-9}$ respectively). These results were supported by luciferase assays, which demonstrated direct binding of the microRNAs to the SATB1 3' UTR, and by western blot analyses, which demonstrated that transient transfection of the BT549 cell lines with pre-miR-7 or pre-miR-155 resulted in a reduction in SATB1 protein levels. It was therefore concluded that these FOXP3-regulated microRNAs target endogenous *SATB1*, thus supporting the hypothesis of Chapter 4: "FOXP3 is able to regulate the expression of *SATB1* by binding to the promoter region of *SATB1* and also by regulating miRs that bind to the 3'UTR of *SATB1*".

A similar quantitative RT-PCR experiment was performed after completion of this thesis, in which the BT549 and MDA-MB-231 breast cancer cell lines were both transiently transfected with pre-miR-155 (Corrigendum Figure 1b). As found in Figure 4.6a of this thesis, transient transfection of the BT549 cell line with pre-miR-155 results in a significant reduction (~45%) of endogenous *SATB1* message levels when compared with the un-transfected parental and control pre-miR-transfected lines. This miR-155-dependent downregulation of SATB1 is also seen in the MDA-MB-231 cell line (~85% reduction in *SATB1* message levels), once again supporting the hypothesis of this thesis that FOXP3-regulated microRNAs target *SATB1*.

The raw Corbett Rotor-Gene data for this experiment can be found on this disc in the document entitled “NJM_corrigendum_Raw data.pdf”. The Microsoft Excel analysis of the data is also located on this disc in the document “NJM_corrigendum_Analysed data.pdf”, as are the calculations performed in Microsoft Excel in which the *SATB1* levels were determined relative to the parental controls (“NJM_corrigendum_Analysed data_relative.pdf”). Together, these documents show that no errors have occurred in the transfer of numbers from the original raw data to the analysed data for Corrigendum Figure 1b.

Overall, it is clear that despite the possibility that there are minor errors in the analysis of the quantitative RT-PCR experiments performed for this thesis, significant evidence from other experiments performed both during and after completion of this thesis suggest that the original hypotheses and conclusions of this thesis are valid.

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Abstract

During their lifetime, 1 in 9 Australian women will be diagnosed with breast cancer, a disease that arises due to mutations and epigenetic modifications to tumour suppressor genes and cancer-promoting oncogenes. This thesis investigates the tumour suppressive role of a transcription factor called Forkhead box Protein 3 (FOXP3) in breast cancer. Little is known regarding its role in the breast and therefore identification of FOXP3-sensitive pathways has the potential to highlight novel targets for breast cancer diagnosis and therapy.

FOXP3 is a 'master regulator' in immunosuppressive T regulatory cells, where it is essential for both cell development and function. It was previously thought that *FOXP3* expression was restricted to these immune cells, however recent studies have identified *FOXP3* expression in breast epithelia, where it has potential tumour suppressor properties. *FOXP3* is mutated or has reduced expression in a significant proportion of human breast cancer samples, and loss of *FOXP3* has been linked to increased mammary tumour formation in animal models. Few targets of FOXP3 in the breast have been identified, but it is known to directly repress the *HER2* and *SKP2* oncogenes while maintaining expression of the *p21* tumour suppressor gene.

A number of groups have shown that in T regulatory cells, FOXP3 regulates a number of small, non-coding RNAs called microRNAs (miRs). Importantly, many studies have reported extensive microRNA deregulation in human diseases, including breast cancer, and

it was therefore hypothesised that similar regulation of miRs by FOXP3 occurs in breast epithelia.

This thesis describes how FOXP3 induces two microRNAs, miR-7 and miR-155, in breast epithelial cells, with these miRs contributing to FOXP3-mediated tumour suppressive activity. One way this is achieved is through co-operation with FOXP3 in a feed-forward regulatory loop to suppress an oncogene called *SATB1*. *SATB1* is highly overexpressed in late-stage breast cancers and promotes metastasis, the final and most fatal stage of breast cancer. This work has established that the *SATB1* promoter is a direct target for FOXP3 repression and that miR-7 and miR-155 target the 3'UTR of *SATB1* for further suppression. Re-introduction of *FOXP3* into breast cancer cells using lentiviral technology results in reduced cell proliferation and invasion potential, supporting a role for FOXP3 as a tumour suppressor.

To further understand the physiological importance of *FOXP3* loss in cancer development, this work also investigated the role of FOXP3 in normal and immortalised breast epithelial cells, with results suggesting that *FOXP3* expression prevents the acquisition of a cancerous phenotype. One way that it may achieve this is by maintaining elevated levels of miR-7 and miR-155. After further investigation, it was found that FOXP3 and miR-7 both have the potential to reduce epidermal growth factor receptor signalling and reduce resistance to apoptosis.

In summary, this work describes a role for FOXP3 and the FOXP3-regulated microRNAs miR-7 and miR-155 in preventing the transformation of healthy breast epithelium to a

cancerous phenotype. One way this is achieved is through a novel feed-forward mechanism by which FOXP3 and FOXP3-regulated miRs work together to suppress the pro-metastatic oncogene *SATB1*. This thesis provides important insight into the tumour suppressive role of FOXP3 in breast epithelia and with further investigation, this new knowledge may form the basis for the development of a novel and effective targeted breast cancer therapeutic.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Natasha Jacqueline McInnes and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Abbreviations

ºC	degrees Celsius
Δ3	*FOXP3* isoform lacking exon 3
3'UTR	3 prime untranslated region
µg	microgram
µL	microLitre
µM	microMolar
Ab	Antibody
APC	Antigen Presenting Cells
ATF-2	Activating Transcription Factor 2
BC	breast cancer
BME	Basement Membrane Extract
bp	base pairs
BR	binding region
Breg	Regulatory B cell
BSA	Bovine Serum Albumin
cDNA	complimentary DNA
ChIP-on-chip	combination of Chromatin Immuno-Precipitation and microarray technology
cPPT	central polypurine tract
CSC	cancer stem cell
Ct	cycle threshold
dH_20	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal Growth Factor Receptor

EMT	Epithelial-to-mesenchymal transition
ER	Estrogen Receptor
ERBB2/HER2	Human Epidermal growth Factor Receptor 2
FACS	Fluorescence activated cell sorter
FCS	Foetal Calf Serum
FL	full length
FOXP3	Forkhead box Protein 3
FKH	Forkhead
g	gravitational force
gDNA	genomic DNA
GFP	Green Fluorescent Protein
HAT	Histone Acetyl-Transferase
HDAC	Histone deacetylase
HER2	Human Epidermal growth factor Receptor 2
HMEC	Human Mammary Epithelial cell line
HoxD10	Homeobox D10
IDC	Invasive ductal carcinoma
IDC-NOS	Invasive ductal carcinoma not otherwise specified
IFN	Interferon
Ig	Immunoglobulin
ILC	Invasive lobular carcinoma
IPEX	Immune dysregulation Polyendocrinopathy Enteropathy X-linked disease
IRES	Internal Ribosome Entry Site
IRS	insulin receptor substrate
iTreg	inducible T regulatory cell
Kb	Kilobase
KO	knockout
L	Litre
LB	Luria broth
LPS	Lipopolysaccharide

LV	lentivirus
LZ	Leucine zipper
M	Molar
MaSC	mammary gland stem cell
MCS	multiple cloning site
MDSC	Myeloid-derived suppressor cell
mg	milligram
miR	microRNA
mL	millilitre
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
mTor	mammalian target of rapamycin
MW	molecular weight
n	sample size
NaCl	sodium chloride
NC	nitrocellulose
NES	nuclear export sequences
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear factor kappa B
NLS	nuclear localisation sequences
nM	nanoMolar
ns	not significant
nTreg	natural T regulatory cell
o/n	overnight
PAK-1	p21-activated kinase
PARP-1	Poly ADP-ribose Polymerase 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	progesterone receptor

PRE	post-transcriptional regulatory element
P/S	Penicillin/Streptomycin
RISC	RNA-induced silencing complex
ROR	Retinoic acid receptor-related orphan receptor
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RRE	Rev-responsive element
RSV	Rous sarcoma virus promoter
RT	room temperature
RT-PCR	Reverse transcription real time polymerase chain reaction
SATB1	Special AT-rich binding protein 1
SD	standard deviation
SEM	Standard error of the mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
SKP2	S-phase Kinase Protein 2
STAT3	Signal Transducer and Activator of Transcription 3
TCF4	transcription factor 4
TDLU	Terminal Ductal Lobular Units
TGFβ	Tumour growth factor β
Th	T helper cell
TIP	Tat-interactive protein
TLDA	Taqman Low Density Array
TN	Triple negative
Treg	T regulatory cell
TSA	Trichostatin A
VEGF	Vascular Endothelial Growth Factor
WT	wildtype
ZF	Zinc finger

CHAPTER 1: LITERATURE REVIEW

1.1 Overview

Breast cancer is one of the most significant contributors to cancer-related death for Australian women (BCNA, 2010). It is a disease caused by the accumulation of mutations and epigenetic changes in both tumour suppressor genes and cancer promoting oncogenes (Knudson, 2001), with most death occurring once tumours have spread to secondary organ sites in a process known as metastasis (Han et al., 2008, Polyak, 2007). Cancer is a highly heterogeneous disease, and therefore to better understand the disease and treat breast cancer patients, it is essential that breast cancer subtypes are further characterised, allowing for the development of new targeted therapies (Bertucci et al., 2012). There is now evidence that the transcription factor FOXP3, a master regulator of T regulatory (Treg) cells, acts as a tumour suppressor in breast epithelial cells (Zuo et al., 2007a, Zuo et al., 2007b), however the genes and pathways regulated by FOXP3 in epithelial cells remains largely unexplored. Genome-wide studies using ChIP-on-chip technology have identified potential targets of FOXP3 in human and mouse Treg cells (Fontenot et al., 2003, Sadlon et al., 2010, Marson et al., 2007), many of which appear to also be targets of FOXP3 in breast epithelia (Katoh et al., 2011). A number of microRNAs were also found to be regulated by FOXP3 (Sadlon et al., 2010), which is of interest as microRNAs are often deregulated in human diseases, including breast cancer (Croce, 2009). It is therefore possible that microRNAs are involved in the tumour suppressive function of FOXP3. The work in this thesis aims to investigate the contribution that microRNAs make to the tumour suppressor function of FOXP3.

1.2 Breast cancer

1.2.1 Cause and progression

Cancers result from an accumulation of mutations and epigenetic changes that can either be genetically inherited or spontaneously develop (Jovanovic et al., 2010, Esteller, 2008, Peltomaki, 2012). Some of these genetic changes confer growth advantages to a cell, contributing to the 6 hallmarks of cancer: evasion of apoptosis, limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis and tissue invasion/metastasis (Hanahan and Weinberg, 2011).

All cancers can be classified into two different groups- hereditary/familial cancers or sporadic cancers (Stratton and Wooster, 1996, Kenemans et al., 2004). Hereditary cancers present with an earlier age of onset of the disease, and first-degree relatives of those carrying a dominant mutation have a high risk of also developing disease (van der Groep et al., 2011). Familial cancers have no specific pattern of inheritance, although there are normally more cancer cases within a family that appear to result from a common genetic background or common lifestyle factors (Vargas et al., 2011). The underlying mutations causing a significant proportion of hereditary/familial breast cancers have now been identified, including *BRCA-1, BRCA-2* and *Rab51c* (Meindl et al., 2011). On the other hand, sporadic cancers generally arise later in life due to the accumulation of somatic mutations within the tissue (Tsuda, 2009). There is no apparent difference in the type of genes linked to hereditary vs. sporadic cancers, with mutation of genes that are associated with hereditary cancers (such as *BRCA1/2* and *Rab51c*) also frequently mutated in sporadic cancer subtypes (Meindl et al., 2011). Sporadic cancers are the most common form of the disease, comprising ~80% of all reported cases (Pavelic and Gall-Troselj, 2001).

The two key events that are responsible for the formation and progression of cancers are the activation of tumour promoting oncogenes and the silencing of tumour suppressor genes (Knudson, 2001). Normal cells that are subjected to a number of mutations in cancer promoting oncogenes or tumour suppressor genes can eventually become hyperplastic, with growth no longer restricted due to escape from senescence and contact inhibition, resulting in cell numbers increasing at an abnormally fast rate (Fidler, 2003). Further mutations to oncogenes and tumour suppressors can then go on to increase the abnormal proliferation of cells, resulting in the production of tumour-like growth and development of a solid, neoplastic structure (Fidler, 2003, Russo et al., 2008). Currently it is hypothesised that a small number of critical genetic events that result in the activation of oncogenes and/or inactivation of tumour suppressor genes precedes each stage of tumourigenesis (Knudson, 2001, Peltomaki, 2012). These critical mutational changes function on a background of a large number of additional genetic changes within the cancer genome and altered cellular microenvironment (Knudson, 2001, Peltomaki, 2012, Hanahan and Weinberg, 2011, Floor et al., 2012). Extensive sequencing of cancer genomes has allowed the characterisation of mutations into two broad subgroups- driver and passenger mutations (Nik-Zainal et al., 2012, Stephens et al., 2012). Driver mutations are those that give a selective advantage to a clone in a microenvironment by either increasing cell survival or cell division, and tend to lead to clonal expansions. Passenger mutations do not affect the survival or the ability of a clone to divide on their own, but may still be involved in clonal expansion, as they are present in the context of driver mutations (Nik-Zainal et al., 2012, Stephens et al., 2012).

In aggressive cancers, metastasis is the final step in the progression of solid tumours, and is the most common cause of cancer-related death in patients; however the mechanisms

responsible for this process are still poorly understood (Han et al., 2008, Fidler, 2003, Russo et al., 2008). Metastasis is a 5-step process, which involves the invasion of tumour cells from the primary site into adjacent tissue, followed by intravasation into the circulatory system, survival in the blood stream and extravasation into distant organs/tissues (Fidler, 2003, Russo et al., 2008). Once colonisation of distant organs/tissues has occurred, cells have the ability to once again grow and proliferate, eventually developing into secondary tumours. Gene expression analyses have identified profiles that are associated with disease progression, including groups of genes (such as growth factors and genes involved in Epithelial-to-Mesenchymal transition) whose expression patterns can be used to predict the risk of metastasis (Wesolowski and Ramaswamy, 2011).

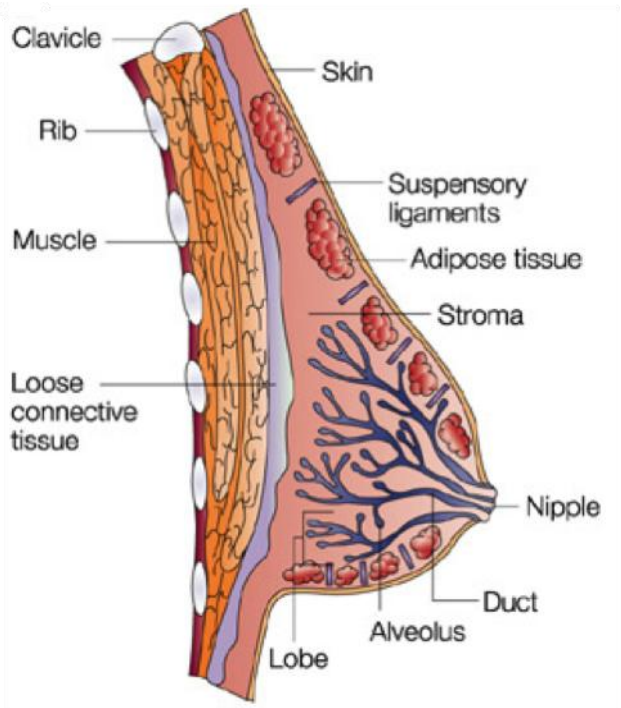
1.2.2 Impact

Breast cancer is the second-largest contributor to cancer-related death in the world after lung cancer. Australian women have a 1 in 9 lifetime risk of developing breast cancer, with this risk increasing with age (BCNA, 2010). Although more often associated with women, breast cancer can also occur in males, although this is extremely rare. Even though there is an overall decreased survival rate in males with breast cancer compared with females, this more likely reflects late diagnosis of the disease and an increase in unrelated, age-associated health issues rather than a fundamental difference in the disease (de Ieso et al., 2012).

1.2.3 Structural anatomy of the breast

The breast tissue is a complex network (Figure 1.1) comprising of epithelial, stromal and adipose cells, and connective tissue that functions to produce milk during lactation

Figure 1.1 ***The structural anatomy of the human breast***



The breast is a complex network comprising of epithelial, stromal and adipose tissue. Image adapted from Ali, S & Coombes, CR (2002) Nature Reviews Cancer. Published with permission.

(Eden, 2010, Wiseman and Werb, 2002). The mammary glands are activated during pregnancy, forming milk-secreting glands that are grouped into lobules that open up to the nipple via lactiferous ducts. In these structures there are 2 key cell types, the luminal cells, which line the apical surface of the breast ducts, and the myoepithelial cells, which surround the luminal cells. Between the lobules there is a mixture of fatty tissue and supportive tissue that is responsible for maintaining the shape of the breast. The most common site of breast cancer development is in the ductal epithelial cells, accounting for 70-80% of all breast cancers, but a significant 10-15% of breast cancers also arise from the lobule epithelial cells. The final 5-10% of breast cancers arise in the connective tissue (Bertos and Park, 2011).

Mammary tissues undergo major changes in growth and architecture during puberty, pregnancy and weaning (Eden, 2010). Terminal ductal lobular units (TDLUs) are the main lobular structures in the breast, and these undergo morphological changes during development. Undeveloped breasts consist of undifferentiated Lob1-type TDLUs (Tiede and Kang, 2011, Russo et al., 2005). During puberty however, TDLUs begin to develop and differentiate, forming Lob2-type TDLUs that have more ductal structures per lobule than Lob1-type TDLUs. During pregnancy, formation of even more ductal structure results in the conversion of Lob2-type to Lob3-type TDLUs, which eventually form the Lob4-type secretory acinar structures required for breastfeeding. After weaning, there is a significant drop in the number of TDLUs, with the majority of the remaining TDLUs of the Lob2-type. This is different in nulliparous women, in which Lob1-type TDLUs are the most predominant, with a small number of Lob2-type structures, but no Lob3 or Lob4-type TDLUs. Once menopause has occurred, all women show predominance for Lob1 structures, regardless of whether or not they have had children. (Russo et al., 2005, Tiede

and Kang, 2011). A population of mammary gland stem cells (MaSC) have been proposed to drive these significant changes in breast growth, restructure and involution during puberty, pregnancy and weaning.

1.2.4 Mammary gland stem cells and cancer stem cells

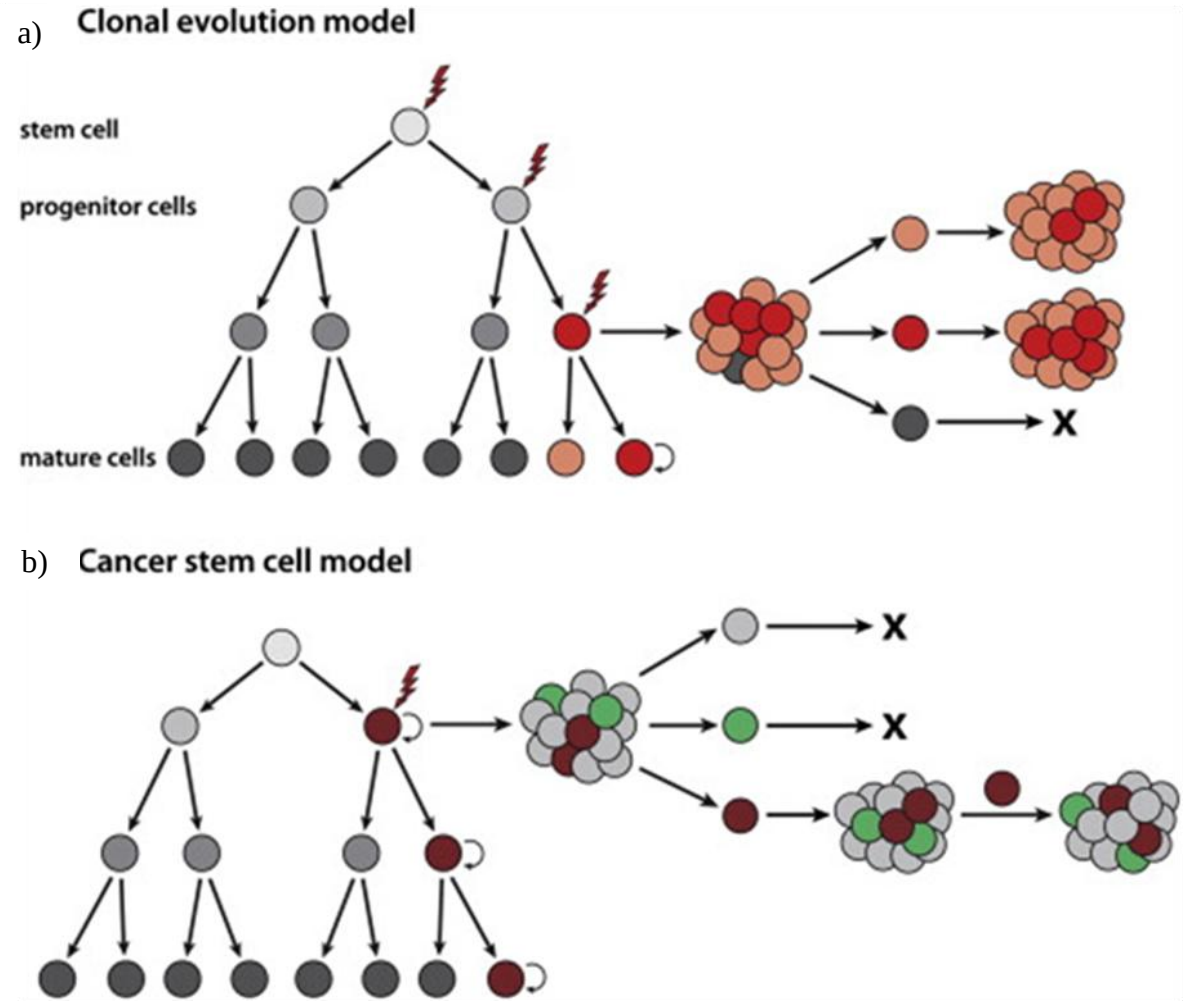
Stem cells are unique in their ability to differentiate into multiple cell types and lineages, while also being able to self-renew in order to maintain a stem cell pool (Shackleton et al., 2006). The existence of adult mouse mammary gland stem cells was first proposed when mammary epithelium transplanted into recipient 3-week-old mouse fat pads was found to be able to differentiate and expand to become a fully functional mammary gland. This ability to reconstitute the mammary gland was not restricted to cells from specific areas of the donor mammary gland, as cells from nearly any location and at any developmental stage had the ability to repopulate the gland (Smith and Medina, 1988). Further investigation showed that this ability was due to the activity of a single cell type, and based on their biological and morphological properties, these cells were classified as mammary gland stem cells (MaSCs) (Visvader and Lindeman, 2006). However, accumulating evidence points to there being a hierarchy in the stem cell/early progenitor cell population, with mouse mammary gland studies finding that both the luminal and myoepithelial cell populations contain unipotent stem cells in addition to multipotent stem cells (Van Keymeulen et al., 2011).

Due to the long life span and self-renewing ability of MaSCs, it has been proposed that these cells are perfect targets for the transformation events that cause the formation of cancers (Kumar and Kutty, 2012, Bonnet and Dick, 1997), leading to the hypothesis that

transformation of MaSCs results in the generation of cancer stem cells (CSCs) that drives the progression of the disease (Fridriksdottir et al., 2011, Tiede and Kang, 2011, Kumar and Kutty, 2012).

There are two hypotheses to account for tumour development and subsequent heterogeneity: the clonal evolution model and the cancer stem cell (CSC) model (Figure 1.2). In the clonal evolution model, it is proposed that any cell within the tumour has the ability to propagate the tumour. In this model, intra-tumour heterogeneity arises due to competition amongst cancer cells that have accumulated different mutation profiles, with the tumour cells carrying a selective advantage driving cancer progression (Lindeman and Visvader, 2010, Polyak, 2007, Visvader, 2011). The development of therapeutic resistance, a common setback in cancer treatment, can be viewed as a therapy-induced selection pressure driving the dominance of resistant clones (Polyak, 2007). Evidence to support the clonal evolution model was found when studies were unable to identify the CD61/beta3 integrin CSC-specific cell marker in a homogenous mammary tumour mouse model (Lindeman and Visvader, 2010). The cancer stem cell model proposes a hierarchy amongst tumour cells that mirrors the relationship observed between normal stem cells and their differentiated progeny. In this model, a fraction of cells within the tumour have stem cell-like characteristics, and it is these cells that are essential for tumour development, maintenance and growth. CSCs are proposed to persist in tumours as a distinct population that maintain themselves through self-renewal, while aberrantly differentiated progeny contribute to overall tumour bulk. Such cells are proposed to be responsible for relapse and metastasis. This model suggests that heterogeneity is caused by aberrant differentiation of committed daughter cells (Lindeman and Visvader, 2010, Visvader and Lindeman, 2012).

Figure 1.2 ***Two models for tumour development and heterogeneity***



a) The clonal evolution model, in which heterogeneity arises due to the diversity of cells present within a tumour. Mutations arise in tumour cells, conferring a growth advantage. A cell (red) that has acquired multiple mutations can produce a dominant clone. Tumour cells (red and orange) that arise from this dominant clone have similar capacity to form tumours. Other derivatives (grey cells) can lack tumorigenicity. b) The cancer stem cell model. A mutation in MaSCs or a progenitor cell (brown) can give rise to tumour cells with stem cell-like properties, in particular the ability to self-renew. Additional mutations in these cells cause heterogeneity in their differentiated progeny (grey and green cells). Some cells may lack tumourigenity due to stochastic events (X). Image from Visvader J.E. & Lindeman G.J (2012) *Cell Stem Cell.* Published with permission.

Cancer stem cells are highly resistant to drugs (Polyak, 2007), most likely due to their distinct molecular profile from bulk tumour cells (Singh and Settleman, 2010) and the fact that over time these cells can become quiescent (Williams, 2012). Although it seems more likely that CSCs arise due to transformation of a normal MaSC or an early progenitor, it is also possible that CSCs arise when a terminally-differentiated tumour cell acquires stem cell-like properties, such as an increased capacity to self-renew (Visvader and Lindeman, 2012). Importantly, it is clear that these two models are not mutually exclusive, as both models support the hypothesis that tumours originate from a single cell that acquires multiple mutations and unlimited proliferative capacities (Polyak, 2007). Evidence supporting a link between these two models was generated using evolutionary models to study the mutational profile of different cells within individual tumours (Nik-Zainal et al., 2012). These studies found that the common last ancestor of tumour cells is present early in tumour development, with these tumour cells going on to accumulate a large number of mutations. During this process extensive clonal evolution and diversification occurs, but no subtype dominates. Subsequent mutation in one clone allows for proliferative advantage, such that in each of the 21 BC samples examined at the time of diagnosis, one clone was found to dominate >50% of all tumour cells (Nik-Zainal et al., 2012). This suggests that long-lived, slow-dividing cells, such as cells with stem cell-like properties, accumulate mutations until one cell acquires a set of mutations that allows it to dominate. However due to the low sample number, this requires further confirmation.

1.2.5 Risk factors in breast cancer

There are a number of factors that are associated with an increased risk of developing sporadic breast cancer. Some of the most common risk factors that are associated with breast cancers are sex, age, affluence, timing of pregnancy, nulliparity and genetic

predisposition (Key et al., 2001). Women are one hundred times more likely to develop breast cancer than men due to changes to the breast and hormone levels that occur at different life stages, as described above in Section 1.2.3. Another significant contributor to risk of developing the disease is age. Although breast cancer can occur early on in life, a significantly higher proportion of women who are older, especially above the age of 50, are likely to develop the disease. This is thought to be primarily due to the accumulation of DNA damage over time. Other contributing factors include higher levels of estrogen in post-menopausal women, which has been shown to increase cellular proliferation in the breast, thus increasing the likelihood of mutations occurring during cell division (Key et al., 2002, Henderson and Feigelson, 2000).

The percentages of women that are diagnosed with breast cancer are also heavily influenced by geographical and socioeconomic status, with the frequency of cases significantly higher in western populations, most likely due to environmental and lifestyle factors associated with these cultures (Nelson et al., 2012). This increase may also reflect the trend in western cultures for delaying having children until later in life, as a first-time mother over the age of 29 years has an increased risk of developing breast cancer compared with women who have children at an earlier stage in life (younger than 25 years of age) (Iwasaki and Tsugane, 2011). It has also been shown that women who do not have children are more likely to develop breast cancer than those who do have children (Iwasaki and Tsugane, 2011). All of these risk factors have the potential to increase the risk of developing cancer by 1 to 1.5 fold. Interestingly, studies have also shown that breastfeeding reduces the risk of cancer development (Nelson et al., 2012), and it is therefore possible that the increased risk of cancer development observed in western populations is a result of more women choosing to have children at a later age, and/or are

choosing not to breastfeed. Other factors that have also been investigated for their potential role in the risk of developing breast cancer include height, pre- and post-menopausal weight, alcohol intake, physical activity, endogenous and exogenous hormone levels, cigarette smoking and diet. It has been shown that taller height, alcohol intake, heavier post-menopausal weight and smoking all have the potential to increase the risk of developing cancer (Iwasaki and Tsugane, 2011). These factors do not guarantee development of breast cancer, however they can have accumulative effects (Nelson et al., 2012).

1.2.6 Subtypes and heterogeneity

Breast cancers are currently classified into different subtypes based on their grade, location, expression of histopathological markers, morphology and structural organisation (Bertos and Park, 2011). The most common type of cancer, as mentioned earlier in Section 1.2.3, is invasive ductal carcinoma (IDC), with invasive lobular carcinoma (ILC) the next most common histological variety of tumour (Li et al., 2005). IDC can be further sub-categorised into different 'grades'. Well-differentiated (grade 1), moderately-differentiated (grade 2) and poorly-differentiated (grade 3) subtypes are determined as a result of the level of nuclear pleomorphism, glandular/tubular formation and mitotic index displayed by the tumours (Lester et al., 2009). Prognosis worsens with increasing tumour grade, and is also associated with increased risk of recurrence ($p=0.002$) (Nixon et al., 1996). Breast cancers can also be subdivided based on the expression of specific immunopathological markers. Tumours are often divided into three major subtypes, which currently is the first step in determining the most appropriate treatment approach. These subtypes are determined as a result of the presence or absence of estrogen or progesterone receptors (ER & PR respectively), amplification of human epidermal receptor 2 (*HER2*) or are classified

as triple negative (TN) if they are negative for ERs, PRs and HER2 amplification (Higgins and Baselga, 2011). The TN breast cancers are considered to be the most life threatening, as they lack a known therapeutic target, complicated further by the fact that a large proportion of these cancers appear to develop in women containing germline BReast CAncer gene 1 (BRCA1) mutations. Those patients carrying *BRCA1* and *BRCA2* gene mutations have a significantly increased risk of developing cancer, and also have poorer prognosis (Narod and Foulkes, 2004).

The majority of breast cancers originate in the lobular or ductal cells of the milk-producing glands. In these structures there are two main cell types: the inner luminal cells, which are surrounded by myoepithelial cells. Basal myoepithelial cells surround the luminal cells and have both muscle and epithelial properties, while luminal cells line the apical surface of the normal breast ducts and have secretory properties (Bertucci et al., 2009). Most breast cancers are thought to arise from luminal cells, and can be categorised into Luminal A and Luminal B cancers based upon gene expression signatures (see below). The most common form is the Luminal A subtype, comprising 45% of all breast cancers (Bertucci et al., 2009). These cancers are often low grade, differentiated tumours that express hormone receptors. In general, Luminal A cancer patients have relatively good survival rates, respond well to hormone therapy, but do not respond well to chemotherapy (Bertucci et al., 2009). Luminal B cancers are very similar to Luminal A cancers, but often have a higher tumour grade due to increased proliferation and poorer prognosis. Basal cancers represent ~15% of all breast cancers, and have a high tumour grade, are proliferative and are negative for hormone receptors. There are also other, rarer types of breast tumours, including medullary, tubular, apocrine, neuroendocrine, inflammatory, mucinous, comedo, adenoid cystic, metaplastic and micropapillary cancers (Malhotra et al., 2010). The sub-

types of breast cancer can also be associated with patient prognosis, with the worst survival rates given to patients with IDC, ILC, apocrine and medullary carcinomas (Bertos and Park, 2011).

Genomics research has facilitated significant advances in the field of cancer, as genome-wide comparisons can find gross (deletions, amplifications, inversions and translocations) and subtle (altered expression levels/mutations) differences between cancers that may then be targeted for therapeutic benefit. Gene expression profiling of breast cancer cohorts has also led to the identification of multiple molecular subtypes that are associated with ER, PR, HER2, Luminal A/B and basal breast cancers. Molecular subtypes are classified based on the distinct transcriptional signatures, and show a correlation with the immunopathological status of the cancers (Malhotra et al., 2010). Although the gene expression profiles appear to correlate to some extent with the immunopathology of the disease, clustering cancers by studying the gene expression profile derived from bulk tumours does not take into account the intratumoral heterogeneity or mixed phenotypes of individual cells (Bertos and Park, 2011). For example, breast cancers are considered as ER^+ and $HER2^+$ when the ER and HER2 positive cells comprise 1% and 30% of the total breast cancer cell population respectively (Bertos and Park, 2011).

One important new frontier in breast cancer classification involves the investigation of microRNA (miR) expression in breast cancers. MicroRNAs are small, non-coding RNAs that are able to post-transcriptionally regulate target mRNAs by causing mRNA degradation or by blocking translation (Pasquinelli, 2012). MiR expression profiling studies using large cohorts of breast cancer samples have established an apparent link

between miR profiles and molecular subtype, tumour grade and ER status (Zhang et al., 2007, Olson et al., 2009, Lyng et al., 2012, Buffa et al., 2011). Many miR and mRNA expression changes that are observed in breast cancers are a result of epigenetic changes rather than genomic changes (Castaneda et al., 2011, Bertos and Park, 2011) and recently it has been shown that DNA methylation profiles may be a useful tool for the further classification of breast cancers. For example, DNA methylation profiles have the ability to subdivide breast cancer samples into multiple luminal A-enriched and basal-like/HER2 enriched clusters (Kamalakaran et al., 2011). This suggests that methylation profiling may be able to capture additional information beyond molecular and immunopathological status.

1.2.7 Targeting cancer for treatment

The current, widely used techniques for the treatment of breast cancer patients include surgery, hormonal therapy, chemotherapy, radiation and immunotherapy; however in many cases none of these options alone are sufficient to clear the disease (Bertucci et al., 2012, Stebbing and Ellis, 2012). The chance of success is significantly reduced at later stages of breast cancer progression, predominantly due to the development of therapeutic resistance and metastasis. Surgery still remains the best approach for disease-free survival. Chemotherapy is often given in conjunction with surgery, but there are many debilitating side effects, including hair loss, nausea and digestive disturbance due to the toxicity of the agents to all rapidly dividing cells (Stebbing and Ellis, 2012). However, the potential benefit of adjuvant therapies normally outweighs the risk associated with these treatments, often significantly extending patient life expectancy. For example, in a study of 493 patients with stage I to III TN breast cancer, it was found that those who receive adjuvant chemotherapy have a 52% higher survival rate than those who receive neoadjuvant therapy

or no therapy (Kennedy et al., 2010). New adjuvant and neoadjuvant treatments, and new combinations of treatments currently in clinical trials may lead to further improvements in survival rates (Bertucci et al., 2012). It is also of interest to determine if individual genetic information from patients can be used to produce more specific and effective therapies.

Identification of key molecular drivers and pathways in breast cancer progression has led to the development of a number of targeted therapies. Due to the heterogeneity of breast cancer, these therapies are often restricted to a subset of cancers. Current treatment of cancers that express ER and HER2, with agents that target these markers (including the ER antagonist Tamoxifen and the HER2 antibody Herceptin) can be effective; however there is still the problem of the cancers developing resistance (Bertucci et al., 2012). Currently there is no targeted therapy for TN breast cancers in clinical use, although poly adenosine diphosphate ribose polymerase (PARP), epidermal growth factor receptor (EGFR) inhibitors, histone deacetylation inhibitors and anti-angiogenic agents have been shown to have some therapeutic potential (Tate et al., 2012, Griffiths and Olin, 2012). One strategy being developed involves targeting the process of DNA damage repair. One mechanism by which this is achieved is through the inhibition of Poly ADP-ribose polymerase (PARP-1), an enzyme that is critical for the base excision repair of single-stranded DNA breaks (Turner et al., 2005). Inhibitors of PARP are currently in clinical development (including iniparib, olaparib and velaparib), with phase II trials showing that in combination with chemotherapy, iniparib was relatively successful at treating triple negative patients when compared with patients treated with chemotherapy alone (rate of response improving from 16% to 48%, clinical benefit 21% to 62%) (O'Shaughnessy et al., 2011a). However by phase III of the trial, improvement as a result of treatment with the inhibitor was no longer significant, suggesting that patients may have benefited from treatments in combination

with other therapies (O'Shaughnessy et al., 2011b). Another therapeutic strategy involves targeting tumour angiogenesis, one of the hallmarks of cancer (Hanahan and Weinberg, 2000). In particular, extensive research has gone into the monoclonal antibody bevacizumab, which is directed against Vascular Endothelial Growth Factor (VEGF) (Miller et al., 2007). To date, it remains unclear whether triple negative cancers are more sensitive to anti-angiogenesis drugs than others (Bertucci et al., 2012), but they do appear to respond as well to treatment as other cancers (Miller et al., 2007). Intracellular signalling pathways are also key targets in the development of treatment strategies for breast cancer. Some of the current therapies being tested include treatment with inhibitors of epidermal growth factor receptor (EGFR), mammalian Target of Rapamycin (mTOR), ABL and SRC kinases, Nuclear Factor kappa B (NFκB) pathway, Tyrosine Kinase receptors and chemokine receptors (Bertucci et al., 2012). As most of these trials are ongoing, currently little is known about the efficiency and safety of these inhibitors as breast cancer therapeutics. Although these treatments may be effective in many patients with TN breast cancer and demonstrate the benefits of targeting specific mutations, these therapies are likely, as observed for ER and HER2 inhibitors, to eventually fail in many incidences, resulting in cancer relapse (Bertucci et al., 2012). The efforts to develop novel therapeutics are further complicated by the heterogeneity displayed by TN breast cancers, however recent efforts to subdivide TN breast cancers may reveal new therapeutic approaches for subtypes of this broad classification (Bertucci et al., 2012). This underlies the importance of better understanding the heterogeneous nature of breast cancers, to drive discovery of new therapy combinations. By further subdividing cancers, not only will there be a better understanding of the biology and clinical outcome of cancers, but there will also be the potential to identify case-specific diagnostic, prognostic and therapeutic targets with fewer side effects and better survival rates for breast cancer (Bertucci et al., 2012).

1.3 FOXP3

1.3.1 Immune regulation in cancer

Modulation of the immune system at the site of cancers is emerging as a novel hallmark of cancer (Curigliano, 2011). This involves both the direct promotion of tumour growth via inflammation, as well as indirectly assisting cancer cells to evade immune-mediated destruction (Ohm and Carbone, 2002, DiDonato et al., 2012). Multiple mechanisms for evasion of immune regulation exist. These include immune editing (by selecting for cancer clones with lower immunogenicity) (Kim et al., 2007), disabling immune cells through the secretion of immunosuppressive cytokines (such as TGF-β) (Kirkbride and Blobe, 2003) and/or the recruitment or induction of immune cells that have regulatory or immunosuppressive function (Draghiciu et al., 2011). Such regulatory immune cells include T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSC) and regulatory B (Breg) cells (Kosmaczewska et al., 2008, Martin et al., 2012, DiLillo et al., 2010). One of the most important and well-characterised regulatory cell populations is the $CD4^+CD25^+FOXP3^+$ Treg cell population.

1.3.2 *FOXP3* and the regulatory T cell

The functional utility of an immune system is dependent on its ability to react to pathogens whilst preventing immune responses to self and harmless antigens such as food, commensal microorganisms and environmental antigens. This process is termed immune tolerance (Sakaguchi et al., 2008). The immune system is divided into two components, innate and adaptive immunity. Innate immunity is a non-specific defence mechanism that activates shortly after exposure to antigen, while the adaptive immune system is a more complex antigen-specific response (Zwirner et al., 2010). In addition, the adaptive immune system must also contain mechanisms to ensure an appropriate level of immune response

to a pathogen to limit damage to host tissues caused by the immune response itself (Josefowicz et al., 2012). T Regulatory (Treg) cells, a rare subset of $CD4^+$ T helper cells, are an essential component for establishing and maintaining tolerance in the periphery (Sakaguchi et al., 2008). Treg cells function by acting in trans to downregulate or suppress the reactivity of other immune cells of both the innate and adaptive immune system (Fehérvari and Sakaguchi, 2004, Josefowicz et al., 2012). T regulatory cells can be further subdivided into two types based on their ontology. Natural Treg (nTreg) cells develop as a separate $CD4^+$ lineage in the thymus, while inducible Treg (iTreg) cells develop from naïve $CD4^+$ T cells in the periphery under antigen stimulation in the presence of TGF-β and IL-2 (Curotto de Lafaille and Lafaille, 2009, Josefowicz et al., 2012). Emerging evidence indicates that these two types of Treg cells have different and non-redundant roles in the body (Josefowicz et al., 2012, Curotto de Lafaille and Lafaille, 2009, Samstein et al., 2012). More recently, $CD8^+FOXP3^+$ Treg suppressive cells have also been identified, which share many phenotypic and developmental characteristics with the $CD4^+FOXP3^+$ cells, however their ability to suppress is not as pronounced as the $CD4^+CD25^+$ Treg subset (Mayer et al., 2011).

A defining hallmark of Treg cells is the expression of the X-linked Forkhead box protein 3 (*FOXP3*) gene, a member of the forkhead/winged helix transcription factor family (Fontenot et al., 2003, Marson et al., 2007). The essential role of FOXP3 in Treg cells was first highlighted by the identification of mutations in the *FOXP3* gene underlying the Scurfy mouse phenotype and Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome in humans (Bennett et al., 2001, Brunkow et al., 2001). Loss of function mutations in *FOXP3* in the Scurfy mice and IPEX patients results in an early onset, T-cell dependent immune disorder characterised by lymphoproliferation, multi-

organ infiltration and cytokine storm caused by the loss of a functional $CD4^+CD25^+$ Treg cell population (Brunkow et al., 2001, Bennett et al., 2001). In the T cell compartment, high levels of *FOXP3* expression are thought to be restricted to the $CD4^+CD25^+$ Treg population, with subsequent gene targeting and knockdown studies demonstrating high *FOXP3* expression is essential for the development, maintenance and function of both nTreg and iTreg populations (Curotto de Lafaille and Lafaille, 2009). In addition, retroviral expression of the *FOXP3* transgene has been shown to be sufficient to confer suppressive activity to non-regulatory $CD4^+$ T cells and correct the Scurfy phenotype (Fontenot et al., 2003). This has led to the proposal that *FOXP3* is a 'master regulator' gene required for both Treg cell development and function (Fontenot et al., 2003, Buckner and Ziegler, 2008). However, recent evidence has indicated that lower levels of *FOXP3* in T cells can occur without the acquisition of suppressive function (Sakaguchi et al., 2010, Miyao et al., 2012) and this finding, coupled with the identification of *FOXP3* expression in other cell types, suggests that FOXP3 may play a broader role in the body.

1.3.3 $FOXP3^+$ T regulatory cells in cancer

Although *FOXP3*-expressing Treg cells are critical for immune homeostasis and the prevention of autoimmunity, these cells may also have a negative effect on health by down-regulating beneficial immune responses, including anti-tumour responses (Facciabene et al., 2012). Tumour infiltrating $CD4^+CD25^+$ Treg cells and $CD8^+FOXP3^+$ cells have been observed in many tumour sites, where tumour infiltrating Treg cells and $CD8^+FOXP3^+$ cells prevent the immune system from clearing cancerous cells (Kiniwa et al., 2007, Yu and Fu, 2006). Animal studies have also demonstrated that removal of $CD4^+$ Treg cells or blocking Treg cell function results in a significant increase in tumour clearance and animal survival (Teng et al., 2011, Onizuka et al., 1999). In humans, tumour-

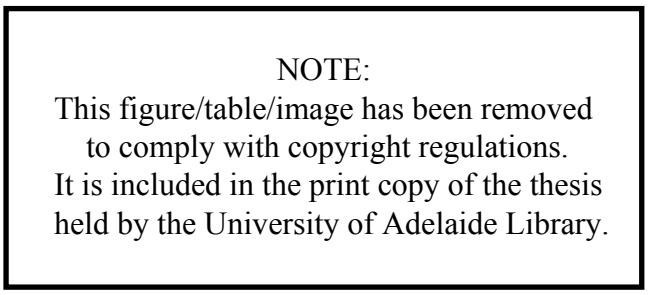
infiltrating Treg cell numbers negatively correlate with patient survival (Curotto de Lafaille and Lafaille, 2009). Critically, it has been found that tumour tissues can promote the conversion of naive T cells into $FOXP3^+$ iTreg cells, which results in their subsequent accumulation at the tumour sites, and a reduction in effector responses (Liu et al., 2007, Nishikawa et al., 2003).

1.3.4 The *FOXP3* gene

Using bioinformatics and known secondary structures of functional domains, homology searches have identified four putative functional domains within FOXP3 (Buckner and Ziegler, 2008). These include an N-terminal domain, zinc finger domain, leucine zipper domain and the forkhead domain (Figure 1.3). The clustering of point mutations in these putative domains in IPEX patients suggests that they are likely to be functionally critical (Ziegler, 2006).

The forkhead domain is a defining feature of the forkhead/winged helix family of transcription factors, and has been shown to be both necessary and sufficient for DNA binding, nuclear import and FOXP3 interaction with other transcription factors, including NFAT (Wu et al., 2006, Lopes et al., 2006). The leucine zipper domain has been established as both necessary and sufficient for homodimerisation of FOXP3. Disruption of this domain either through deletion or point mutation has been shown to block the ability of FOXP3 to dimerise and act as a repressor of transcription (Li et al., 2007). Contained within the N-terminal region is a proline-rich repressor domain that is critical for transcriptional repression by FOXP3 (Lopes et al., 2006). A role for the zinc finger domain has yet to be conclusively established, however experimental mutation of the zinc finger

Figure 1.3 ***The FOXP3 gene***



The critical domains of FOXP3 as determined by the location of known IPEX mutations. The locations of missense mutations located within the FOXP3 protein (top) are indicated by black arrows. The mutations that are predicted to affect splicing or stability (red arrows) or mutations that generate frameshifts (blue arrows) are indicated on the schematic of *FOXP3* mRNA (bottom). The Δ3 isoform of *FOXP3* lacks exon 3. The exons are colour-coded as per the protein schematic, and pale blue regions indicate coding regions of unknown functions. Image adapted from Ziegler, S.F. (2006), *Annu Rev Immunol*. Published with permission.

only mildly blocks the repressive activity of FOXP3, indicating that it does not play an essential role in the repressor activity of FOXP3 (Lopes et al., 2006, Zeng et al., 2011).

There are two isoforms of *FOXP3* that are expressed in human Treg cells; the full length (FL) isoform, and an isoform lacking exon 3 of the gene (Δ3 isoform). The mRNA encoding these two isoforms and the proteins themselves are expressed approximately equally in human Treg cells, however interestingly, the Δ3 isoform is not found in the mouse (Allan et al., 2005). Emerging data suggests that functional differences exist between these isoforms, and that this may drive cell type-specific responses. For example, exon 3 is located within the N-terminal repressor domain of *FOXP3*, and disruption of this domain decreases FOXP3 repressive activity (Lopes et al., 2006). In addition, this region is normally involved in binding to retinoic acid receptor-related orphan receptor (RORα), with removal of exon 3 by alternative splicing sufficient to disrupt this interaction and inhibit FOXP3-mediated repression of RORα target genes (Du et al., 2008).

Two common themes have emerged from these investigations of the molecular mechanisms of FOXP3 function in Treg cells. The first is that FOXP3 physically interacts with a number of other transcription factors, which can modify the transcriptional programs of those transcription factors. One example of a key transcription factor that is regulated by FOXP3 is Nuclear Factor of activated T cells (NFAT) (Wu et al., 2006). NFAT is a key transcription factor involved in T cell activation and anergy via regulation of T cell activation-associated genes, forming cooperative complexes with the AP family of transcription factors (Bettelli et al., 2005, Wu et al., 2006). NFAT is able to interchangeably interact with a number of other regulatory proteins, and has been shown to

play a role in both T cell activation and in the suppressive activity of Treg cells. A cooperative complex composed of NFAT and FOXP3 mediates this, with this interaction leading to down-regulation of IL-2 and upregulation of the Treg markers CTLA4 and CD25 (Wu et al., 2006). Treg cells that have this NFAT-FOXP3 interaction disrupted via mutation of the key FOXP3 binding regions display a reduced ability to regulate target genes, and have also been shown to have a reduced suppressive capacity (Wu et al., 2006). The second theme that has emerged from studies of FOXP3 in Treg cells is that FOXP3 modifies gene expression through epigenetic modification, including through the formation of complexes with chromatin modifiers that have been linked to changes in chromatin (Bettini et al., 2012, Josefowicz et al., 2012). One example of these complexes is the interaction that FOXP3 has with a histone acetyltransferase (HAT) TIP60 and Class II histone deacetylases (HDAC), HDAC7 and HDAC9 (Li et al., 2007). FOXP3 binding to acetyltransferases and deacetylases has been associated with changes in histone acetylation and methylation (Zheng et al., 2007, Katoh et al., 2011). The interaction between FOXP3, HAT and HDA7 is required for the regulation of IL-2 in T cells; however the interaction of FOXP3 with HDAC9 is more complex. While interaction between FOXP3 and HDAC9 is antagonised by T cell stimulation, it can be restored by the protein deacetylation inhibitor trichostatin A (TSA), indicating that this interaction may in fact be a complex dynamic aspect of T suppressor regulation that responds to T cell receptor signals (Li et al., 2007, Zhang et al., 2012).

1.3.5 Expression of *FOXP3* outside of the immune system

Up until a few years ago it was believed that *FOXP3* expression was restricted to the $CD4^+CD25^+$ Treg cell population. However, there is now evidence that *FOXP3* is expressed in other T cell lineages, as well as non-haematopoietic cell types, including

human breast, prostate and lung epithelia (Wang et al., 2009, Zuo et al., 2007b, Tao et al., 2012), at both a message and protein level. In addition, *FOXP3* expression at both the message and protein levels has also been detected in a number of cancer cell lines and primary tumour cells (Ebert et al., 2008, Karanikas et al., 2008).

1.3.6 FOXP3 as a tumour suppressor in breast epithelial cells

A link was first made between FOXP3, tumour suppression and breast epithelial cells when heterozygous *Foxp3* knockout mice were reported to show high frequencies of spontaneous cancer development, particularly in the mammary gland (Zuo et al., 2007b). Extended observations of female mice heterozygous for the scurfy mutation ($Foxp3^{sf/wt}$) revealed a high incidence of age-dependent spontaneous cancer, 60% of which were mammary cancers. These mice were also found to be more susceptible to chemical-induced carcinogenesis (Zuo et al., 2007b).

Laser-capture microdissection followed by qPCR and immunohistochemical staining demonstrated that Foxp3 message and protein was expressed in normal epithelial cells derived from mammary glands of wildtype and heterozygous *Foxp3* mice, with expression significantly reduced in the cancerous mammary epithelia from the heterozygous mice (Zuo et al., 2007b). Contamination by T cells was eliminated by qPCR for T cell-specific CD3. Critically, tumour cells, but not the adjacent normal breast tissue from these mice, showed evidence of skewed X-inactivation, with all cancerous cells found to be expressing the $Foxp3^{SF}$ mutant allele while the $Foxp3^{WT}$ allele was found to be on the inactive X chromosome. Normal tissue displayed a mosaic pattern of $Foxp3^{sf}$ and $Foxp3^{WT}$ expression consistent with random X inactivation (Zuo et al., 2007b). This suggests that parental,

normal epithelial cells lacking *Foxp3* expression were more susceptible to malignant transformation. Analysis of *Rag*$^{-/-}$ mice that lack T cells has confirmed that *Foxp3* expression occurs in breast and other epithelial cells. Immunohistochemistry and real-time PCR analysis identified *Foxp3* expression at both the message and protein levels in breast, lung and prostate epithelial cells, with specificity of these experiments confirmed by the ablation of this expression in mice carrying the *Foxp3*^{SF} mutation (Chen et al., 2008b). Introduction of a *GFP* open reading frame into the 3'UTR of the *Foxp3* locus, which tags any cell expressing *Foxp3* with *GFP*, confirmed that the locus is broadly transcribed in the epithelial cells of many different organs (Chen et al., 2008b). Of interest, *FOXP3* levels are significantly lower in epithelial cells when compared with Treg cells, with a 3 to 100-fold reduction in *FOXP3* transcript levels observed (Chen et al., 2008b).

Further support for a physiological role for FOXP3 in epithelial cells was generated by studies in human and mouse breast cancer cell lines, where *FOXP3* was found to be expressed at a much higher level in normal mammary epithelial cells (HMECs) and in the immortalised, non-tumourigenic breast epithelial cell line MCF10a, but was significantly reduced or lost in breast cancer cell lines (Zuo et al., 2007b). Interestingly, while HMECs express both the full length (FL) and Δ3 isoforms of *FOXP3*, none of the breast cancer cell lines tested, including MCF10a, expressed the FL transcripts (Zuo et al., 2007b). This suggests that loss of FL *FOXP3* occurs in cells progressing from a normal cell status to a disease cell status. Analysis of primary breast tumours by fluorescence *in situ* hybridisation found that in 223 samples tested, the 28 cases that had deletions within the X chromosome were all missing the *FOXP3* locus (Zuo et al., 2007b). Sequencing of 65 formalin-fixed or frozen breast cancer samples found that 36% of the total samples contained somatic mutations of *FOXP3*, with 38% of these lacking the wildtype allele of *FOXP3*. These

mutations have been shown to cluster in the known functional domains of FOXP3 (Zuo et al., 2007b), and therefore they are likely to disrupt function. In support of this, many of these *FOXP3* mutations have also been identified in the human disease IPEX (Figure 1.3).

FOXP3 has also been shown to have growth suppressive and anti-metastatic effects in *in vitro* and *in vivo* models. Importantly, re-introduction of *FOXP3* into breast cancer cell lines results in significant growth inhibition, and has pro-apoptotic effects both in an *in vivo* syngeneic BALB/c mouse model injected with *Foxp3*-transfected mammary cancer cell lines and in mouse mammary cancer cell lines *in vitro*, thus further supporting a link between *FOXP3* loss and cancer development (Zuo et al., 2007a, Zuo et al., 2007b). Similar effects have also been observed in human breast cancer cell lines *in vitro* and in xenograft models, indicating that this is not just a phenomenon seen in mice (Heinze et al., 2011, Katoh et al., 2011, Ladoire et al., 2012, Zuo et al., 2007a, Zuo et al., 2007b).

The consequences of *FOXP3* expression on breast cancer prognosis are yet to be fully resolved. Similar to the mouse, Zuo *et al.* observed downregulation of *FOXP3* in 80% of the 600+ breast cancer samples studied when compared with matched normal samples (Liu and Zheng, 2007). Immunohistochemistry performed by Zuo *et al*. found that 80% of normal breast samples expressed FOXP3 in the nuclei of epithelial cells, but less than 20% of the cancerous tissue showed nuclear FOXP3 staining (Zuo et al., 2007b). A tumour suppressive role for FOXP3 was supported by two independent studies by Ladoire *et al.* in which *FOXP3* expression was associated with better overall survival in $HER2^{+}$ breast cancers and in patients treated with anthracycline-based adjuvant chemotherapy (Ladoire et al., 2011, Ladoire et al., 2012). In the first study, FOXP3 status and patient survival was

compared in 103 patients diagnosed with primary invasive $HER2^+$ breast cancers that had been treated with neoadjuvant chemotherapy. FOXP3 expression was observed in breast cancer cells from 57% of the tumours by immunohistochemistry. Importantly, FOXP3 expression in breast cancer cells was associated with better overall survival (p=0.003) and decreased risk of relapse (p=0.005). In a second study by Ladoire *et al.*, expression of FOXP3 in cancer cells was determined by immunohistochemistry of 1097 tumour samples. Patients had received anthracycline-based adjuvant chemotherapy (Ladoire et al., 2012). Of these tumour samples, 37% were found to express FOXP3, with expression associated with better overall survival (p=0.003) (Ladoire et al., 2012). In contrast to the finding of Zuo *et al.* and Ladoire *et al.*, another study found that expression of FOXP3 in primary breast carcinomas of node-positive patients was associated with a poorer prognosis (Merlo et al., 2009). This discrepancy in findings is currently unresolved, but may reflect differences in the patient populations, including HER2 expression status, percentage receiving adjuvant chemotherapy and the type of chemotherapy used. Of note, the large patient cohort in the Ladoire *et al*. 2012 study were all receiving adjuvant chemotherapy (Ladoire et al., 2012), whereas in the Merlo *et al.* study, only 52% were receiving adjuvant chemotherapy and 44% received hormone therapy (Merlo et al., 2009). In addition, the initial studies reporting that FOXP3 has a tumour suppressive role in the breast used different criteria to score FOXP3 positivity in tumours. In the Zuo *et al.* study, a tumour was scored as FOXP3 positive if FOXP3 expression was observed within the nucleus, as observed for normal breast epithelia (Zuo et al., 2007b), while the studies that associated FOXP3 with poor outcome looked at overall expression in the cell, including total cytoplasmic and nuclear expression (Merlo et al., 2009). A change in the subcellular localisation of FOXP3 from the nucleus to the cytoplasm in breast epithelia will inactivate transcriptional function (Magg et al., 2012) as demonstrated by the analysis of mutations in *FOXP3* associated with IPEX and prostate cancer (see below). A number of these

mutations, including some that have also been characterised in breast cancer (Zuo et al., 2007b) have been shown to prevent nuclear localisation. Secondly, it has recently been shown that in addition to nuclear localisation sequences (NLS), FOXP3 has nuclear export sequences (NES) suggesting that FOXP3 activity is regulated by altering its location (Magg et al., 2012). Changes in the balance of import/export of FOXP3 in breast cancer could thus alter FOXP3 activity in the absence of mutations in FOXP3 itself. Further analysis of large cohorts of breast cancer samples controlled for adjunct therapies and sub-cellular location of FOXP3 are required to address this.

1.3.7 FOXP3 in other epithelial cell cancers

Additional evidence suggests that FOXP3 may act as a tumour suppressor in other epithelial tissues, including prostate and ovarian epithelia. Immunohistochemical analysis examining nuclear *FOXP3* expression in 85 human samples of normal prostate epithelia compared with 92 samples of cancerous tissue found that *FOXP3* expression was absent in 68.5% of the cancer samples. In contrast, 100% of normal and benign tissue had clear FOXP3 staining, consistent with a tumour suppressive role of FOXP3 (Wang et al., 2009). In mice, prostate-specific knockout of *Foxp3* resulted in the development of prostate hyperplasia and prostate intraepithelial neoplasia, 2 signs of pre-cancerous lesions (Chrisofos et al., 2007), indicating that Foxp3 is required for the maintenance of normal prostate cell status (Wang et al., 2009). Sequence analysis of matched clinical samples of normal and cancerous prostate epithelia found single base changes to *FOXP3* in 5/20 cases, 4 of which were missense mutations. Importantly, one of these mutations was also reported in breast cancer (Wang et al., 2009, Zuo et al., 2007b), although a recent study suggests that at least in the Korean population, somatic mutations in FOXP3 in prostate cancers may be a rare event (Kim et al., 2011). Similar to breast cancer cell lines, re-introduction of

wildtype *FOXP3* into prostate cancer cell lines was shown to cause significant growth inhibition (Wang et al., 2009).

A correlation has also been found between *FOXP3* expression levels and ovarian cancer development (Zhang and Sun, 2010). Immunohistochemistry of 27 human malignant ovarian tumours and 7 normal ovarian epithelium samples found FOXP3 protein in normal ovarian epithelium, while no or weak expression was detected in tumour cells. Further investigation found that in ovarian epithelial cancer cell lines, re-introduction of *FOXP3* expression resulted in decreased cell growth, inhibited cell cycle associated proteins and decreased cell migration and invasion (Zhang and Sun, 2010). Together these data suggest that *FOXP3* expression and perhaps location (nuclear vs. cytoplasmic) and isoform (FL vs. Δ3) of *FOXP3* are critical determinants of tumour suppressor function by FOXP3 in several epithelial tissues.

1.3.8 Molecular mechanisms of FOXP3 tumour suppressor activity in cancer

FOXP3 is able to function as both a transcriptional activator and a repressor in T cells and epithelial cells (Marson et al., 2007, Pederson, 2007, Zuo et al., 2007a, Zuo et al., 2007b). Genome-wide ChIP-on-chip studies have identified potential FOXP3 targets in both human and mouse Treg cells (Marson et al., 2007, Sadlon et al., 2010, Zheng et al., 2007). More recently, FOXP3 binding regions have also been investigated by ChIP-seq in a human breast cancer cell line, MCF-7, engineered to ectopically express *FOXP3* under inducible Tet-control (Katoh et al., 2011). Comparison of the MCF-7 ChIP-seq data with the results from the human Treg studies showed that 58.5% of the targets overlap (Katoh et al., 2011,

Sadlon et al., 2010), consistent with FOXP3 regulating both conserved and tissue-specific targets.

In keeping with a tumour suppressor function in breast epithelium, FOXP3 has been shown to directly repress the expression of several well-established oncogenes (such as *HER2*, *SKP2* and *cMYC*), while at the same time maintaining expression of tumour suppressor genes (including *p21* and *LATS2*). As described in section 1.2.6, overexpression of *HER2* in breast cancer defines a molecular subtype of cancers, and is amplified in 20-30% of invasive breast cancers. HER2 amplification has also been linked to metastasis and reduced overall survival (Hurvitz et al., 2012). In the *Foxp3*^{sf} mouse model, inactivating mutations in *Foxp3* resulted in the overexpression of the murine homologue of *HER2*, *Erbb2*. Conversely, *Foxp3* overexpression via transient transfection of mouse mammary cancer cell lines led to a significant decrease in *Erbb2* expression. Direct binding of Foxp3 to the promoter region of the *Erbb2* gene and down-regulation of promoter activity, as determined by chromatin immunoprecipitation (ChIP) and promoter reporter assays respectively, indicated that Foxp3 was able to directly negatively regulate the *Erbb2* gene (Zuo et al., 2007b). A significant reduction in tumour growth *in vivo* and significantly prolonged survival was seen in mice transplanted with *FOXP3*-expressing TSA cells compared with controls. Similarly, in 50 formalin-fixed and 15 frozen matched normal and cancerous human breast samples, *FOXP3* levels also negatively correlated with levels of *HER2*, supporting the hypothesis that FOXP3 may exert its tumour suppressive properties in part by regulating *HER2* (Zuo et al., 2007b). Consistent with FOXP3 involvement in regulating *HER2*, *FOXP3* expression in human tumour cells was found to be an independent prognostic factor for better patient outcomes in HER2^{+} cancers treated with chemotherapy (Ladoire et al., 2011).

FOXP3 has also been shown to repress transcription of the S-phase kinase-associated protein 2, *SKP2* (Zuo et al., 2007a). *SKP2* has maximal expression at the S and G2 phases of the cell cycle, and appears to primarily be involved in mediating p27 degradation at the G2 phase (Hu and Aplin, 2008). P27 has an essential role in regulating cell cycle progression, and must be degraded for entry into and progression of S-phase (Stacey, 2010). In animal models, targeted mutation of the *Skp2* gene results in delayed animal growth and cell polyploidy (Nakayama et al., 2000). Increased expression of *SKP2* has been identified in approximately 50% of human breast cancers, particularly in patients suffering from early onset of the disease and those with poor prognosis (Sonoda et al., 2006). A link was first made between *Foxp3* expression and the oncogene *Skp2* in cells derived from wildtype or $Foxp3^{SF/+}$ mice, where inactivation of the *Foxp3* locus coincided with increased levels of *Skp2* expression. A causal link was established between *Foxp3* and *Skp2* levels as transfection of *Foxp3* into mammary cancer cell lines resulted in repression of *Skp2* mRNA levels and an increase in *p27*, a known target of *Skp2* repressive activity (Sonoda et al., 2006, Zuo et al., 2007a). Subsequent ChIP and promoter-reporter assays demonstrated that FOXP3 directly represses *SKP2* transcription. Ectopic expression of *SKP2* from a vector lacking FOXP3 regulatory sites rescued the growth suppression activity resulting from *FOXP3* overexpression in the murine TSA mammary cancer cell line, indicating an important role for FOXP3 in the repression of *SKP2*. This FOXP3-mediated repression of *SKP2* could also be seen in both normal and cancerous human breast clinical samples, however there is not a 1:1 correlation between *FOXP3* expression and the absence of *SKP2*, indicating that other factors contribute to the regulation of *SKP2* (Zuo et al., 2007a). Lastly, in prostate cancer, FOXP3 directly represses the transcription of the *cMYC* oncogene, with inactivation of *FOXP3* both necessary and sufficient for the

overexpression of *cMYC* (Wang et al., 2009). *cMYC* is frequently overexpressed in a number of human cancers (~30%), including ~15% of breast cancers (Liao and Dickson, 2000, Wang et al., 2009). Although not formally proven, this study suggests that in mammary epithelia, FOXP3 may also directly suppress the *cMYC* gene.

On the other hand, FOXP3 has also been reported to positively regulate the expression of the tumour suppressor genes cyclin kinase inhibitor *p21* and the serine/threonine protein kinase *LATS2*. The cyclin kinase inhibitor p21 is responsible for preventing the progression of the cell cycle at the G1/S damage checkpoint by inhibiting cyclin-dependent kinases (Harper et al., 1993). Both *in vivo* and *in vitro* experiments have shown a positive correlation between *FOXP3* and *p21* expression. For example, inducible expression of *FOXP3* in MCF-7 cells results in a 7-fold increase in *p21* transcripts mediated by FOXP3 binding to a site within intron 1 of the *p21* gene, while inactivating *FOXP3* mutations in mouse mammary epithelia results in a 6-fold reduction in *p21* transcripts. This correlation was confirmed in human breast samples, with 66% of $FOXP3^+$ samples also found to be $p21^+$ (Liu et al., 2009a). Studies have shown that loss of *p21* is associated with increased Tamoxifen-mediated growth of breast cancers, presumably through the loss of cell cycle control, and therefore implicates an important role for FOXP3 in the maintenance of *p21* levels (Liu et al., 2009a, Abukhdeir et al., 2008).

Expression of the serine/threonine protein kinase *LATS2* is also positively regulated by FOXP3, with functional FOXP3 binding sites located within the *LATS2* promoter region (Li et al., 2011b). *LATS2* and its target *YAP* genes are members of the HIPPO proliferation and apoptosis pathway, with deregulation of this pathway in human cancers resulting in

increased tumour cell proliferation and increased resistance to apoptosis (Li et al., 2011b). *FOXP3* mutations have also been established as a cause of defective expression of *LATS2* in breast cancer cell lines and prostate cancer human samples. Sequence analysis of 20 matched normal and cancerous human prostate samples also showed that *FOXP3* mutations also result in increased levels of the *YAP* onco-protein, which is also negatively regulated by LATS2 (Li et al., 2011b). Therefore FOXP3 may play a key tumour suppressor role through its interaction with members of the HIPPO pathway.

Recently, FOXP3 has also been shown to positively regulate multiple target genes in breast and prostate epithelia by recruiting the histone acetyltransferase MOF (Katoh et al., 2011). The interaction between MOF and FOXP3 causes the displacement of the histone H3K4 demethylase PLU-1, and increases the permissive histone modifications K4K16 acetylation and H3K4 trimethylation at FOXP3 binding regions of multiple genes positively regulated by FOXP3. Silencing of MOF results in the reduction of gene activation by FOXP3, with mutation of *FOXP3* resulting in reduced nuclear localisation of MOF (Katoh et al., 2011).

Although the mechanisms and pathways involving FOXP3 in breast epithelia have yet to be fully elucidated, together these data suggest that FOXP3 has the ability to control key oncogenes and tumour suppressors in epithelial cells. Interestingly, a significant number of potential FOXP3 targets as determined by the Treg studies of FOXP3 are also involved in breast cancer development. For example, the interaction between FOXP3 and inducible transcription factors such as STAT3/IRF4/NFκB has been documented in Treg biology, with these interactions allowing FOXP3 to sense the signals from tissues and cells in the surrounding microenvironment and tailor Treg responses to particular inflammatory

conditions (Chaudhry et al., 2009, Zheng et al., 2009, Loizou et al., 2011). Interestingly, the overexpression or aberrant regulation of a number of these transcription factors known to interact with FOXP3 in Treg cells (such as RUNX, NFAT, STAT3 and NFκB) have been linked to breast cancer (Mendoza-Villanueva et al., 2010, Foldynova-Trantirkova et al., 2010, Bromberg, 2000, Santini et al., 2011). This suggests that FOXP3 may play a role in the regulation of these transcription factors in normal breast epithelia. In particular, NFκB and STAT3 have important roles in normal mammary biology, such as gland involution and expansion and proliferation of stem cells (Pratt et al., 2009, Scribner et al., 2011). This suggests that FOXP3 may also have a role in breast homeostasis by regulating these processes. Whether an interaction between FOXP3 and these transcription factors is important in the breast has yet to be addressed.

1.3.9 Control of *FOXP3* in epithelial cells

Although an understanding of the mechanisms that regulate FOXP3 in Treg cells is well advanced (Josefowicz et al., 2012), comparatively little is known about the control of FOXP3 in epithelial cells. To date, whether any overlapping regulatory pathways exist between Treg cells and epithelial cells has not been addressed. Several lines of evidence have now linked *FOXP3* expression with DNA damage responses. For example, *FOXP3* is activated as a result of *p53* expression following DNA damage responses (Jung et al., 2010). P53 is a transcription factor with established tumour suppressor function that in normal epithelia induces genes that regulate cell cycle arrest, apoptosis and senescence following exposure to genotoxic and oncogenic stress (Vousden, 2002). *P53* is frequently mutated, resulting in loss of expression or overexpression of mutant forms in human tumours. This leads to disruption of important processes required for cellular control, including apoptosis, proliferation and cell-to-cell signalling (Freed-Pastor and Prives,

2012). This is a complicating side effect that can occur when using genotoxic agents as cancer therapies (Hollstein et al., 1991, Jung et al., 2010).

P53 is known to downregulate expression of the *HER2* and *SKP2* oncogenes (Provinciali et al., 2007, Traub et al., 2006), also known targets for FOXP3 repression (Hu and Aplin, 2008, Jung et al., 2010), suggesting a possible link between FOXP3 and p53. Jung *et al* (2010) have now shown that *FOXP3* expression is induced via a p53-dependent mechanism when DNA-damaging agents are introduced into breast and colon carcinoma cells, suggesting that FOXP3 may also be required for a number of p53-mediated cellular processes (Jung et al., 2010). Subsequently, *FOXP3* induction was shown to participate in the p53 response, as knockdown of *FOXP3* blocked p53-mediated growth inhibitory responses to DNA-damaging agents. Consistent with FOXP3 being involved in DNA damage responses, *FOXP3* expression has been linked with a better outcome in breast cancer treated with chemotherapeutic DNA damaging agents called anthracyclines, a result that may be explained by FOXP3-mediated suppression of *SKP2* (Ladoire et al., 2012) and potentially *p53* induction. It was also found that increased *FOXP3* levels in breast cancer cell lines rendered cells more susceptible to DNA-damage induced cytotoxicity (Ladoire et al., 2012).

Foxp3 transcription has also been linked to activating transcription factor 2 (ATF2) and c-Jun activity (Liu et al., 2009b), with both ATF2 and c-Jun found to interact with an enhancer within intron 1 of the *Foxp3* locus. Treatment of mammary cancer cells with Anisomycin, a potent activator of both *ATF2* and *c-Jun*, resulted in the induction of *FOXP3* expression (Liu et al., 2009b). This was confirmed by targeted mutation of *ATF2*,

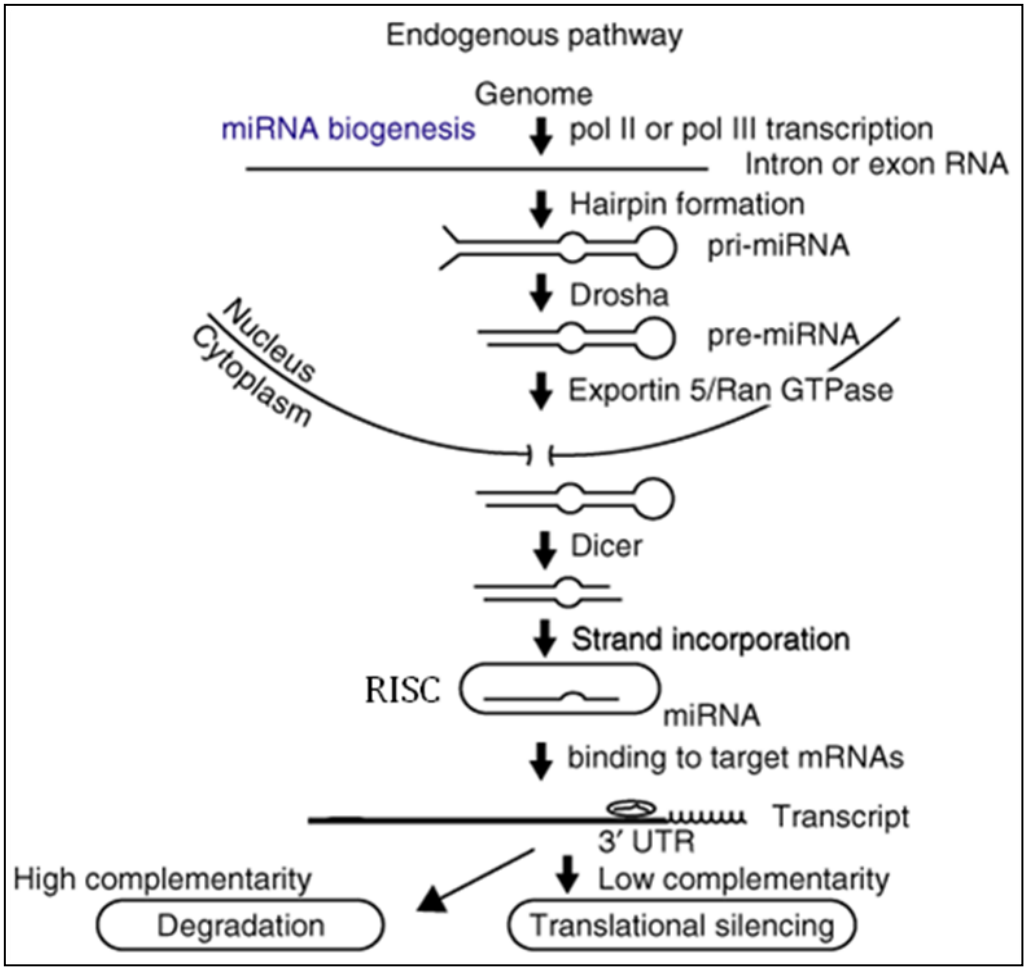
which prevented the expression of *FOXP3* in normal epithelial cells. Importantly, the ATF2-FOXP3 pathway was shown to be critical for Anisomycin-induced apoptosis of breast cancer cells (Liu et al., 2009b), thus supporting a role for FOXP3 as a tumour suppressor.

1.4 MicroRNAs: FOXP3 targets in epithelia

1.4.1 MicroRNA biogenesis and function

MicroRNAs (miRs) are a group of small, non-coding RNAs that are approximately 22 nucleotides in length. MicroRNAs are becoming increasingly recognised as significant regulators of gene expression in a wide variety of cell types, playing roles in a number of critical biological processes (Pasquinelli, 2012). MiRs have the potential to recognise a large number of target genes, with each miR on average able to target approximately 200 different genes (Krek et al., 2005). MicroRNAs, whose loci are intergenic or are within the introns of coding genes, are transcribed by either RNA polymerase II or RNA polymerase III. They are derived from transcripts that fold back onto themselves to form longer, hairpin structure precursors called pri-miRs. Within the nucleus, these pri-miRs are processed to a shorter stem-loop structure by the enzyme Drosha, forming pre-miRs. These pre-miRs are transported from the nucleus via Exportin 5/Ran GTPase, after which they are further processed by the enzyme Dicer, which removes the stem-loop structure, prior to loading onto an Argonaute protein within the RNA-induced silencing complex (RISC) (Figure 1.4) (Zhang et al., 2007). These effector complexes are then directed to a specific site, usually within the 3'UTR of a target mRNA, by hybridisation to complementary ‘seed’ sequences between the miR and mRNA. Recent evidence has suggested that some mRNA targets are more enriched for miR seed sequences than others (Wang et al., 2010).

Figure 1.4 ***microRNA biogenesis***



MicroRNAs (miRNA) are transcribed from DNA into primary transcripts (pri-miRNA) before being processed into pre-miRNA, a short, stem-loop structure. Pre-miRNAs are transported from the nucleus, where they are processed into mature miRNAs through the interaction with the Dicer endonuclease. This initiates the formation of the RNA-induced silencing complex (RISC). The single stranded miRNA can then bind with either high or low complementarity to a target mRNA, resulting in degradation of the target or translational silencing. Image adapted from Hildebrand, MS et al. (2008) Molecular Therapy. Published with permission.

Although miR seed sequences are important for determining binding characteristics of some miRs, binding mechanisms can be more complex, depending on target structure, location and the cellular environment (Wang et al., 2010). Once bound to target mRNA, miRs will direct translational repression and/or mRNA destabilisation. The degree of complementarity between the miR and its target mRNA is believed to determine the fate of the target, with extensive base pairing generally favouring mRNA degradation (Pasquinelli, 2012). The destabilisation of mRNA occurs due to the miRs inducing de-adenylation of the target mRNA, triggering de-capping of the message and resulting in an increased speed of degradation by standard mRNA turnover processes. De-adenylation of the target mRNA has also been implicated in reducing translation initiation via Poly-A binding proteins, however this requires further investigation (Brook and Gray, 2012).

Recently, experiments involving cross-linking and Argonaute immunoprecipitation together with high throughput sequencing (CLIP-seq) have been used to determine the targets of human and mouse microRNA (Yang et al., 2011). From this analysis, 400,000 predicted miR-target regulatory relationships were identified, with roles in many biological processes, including cell apoptosis, development, differentiation and proliferation (Yang et al., 2011). Over half of the human transcriptome is predicted to be a target of miR regulation, with miRs appearing to have roles in nearly every major gene cascade. Given the extent of regulation that miRs can provide, and their involvement in important regulatory pathways, it is not a surprise that disruption of miR function can contribute to a number of human diseases (Pasquinelli, 2012). Recently, there has been increased interest in the interaction between transcription factors, miRs and their targets. Evidence has now shown that miRs can be involved in feedback or feed-forward regulatory loops (Eduati et al., 2012, Re et al., 2009, McInnes et al., 2012), with each type of loop producing different

outcomes. An example of a positive feedback loop involves a transcription factor upregulating a target that then upregulates the transcription factor itself. These positive feedback loops produce robust switches by causing an 'all or none' outcome (Herranz and Cohen, 2010). An example of a negative feedback loop involves a transcription factor that upregulates a miR, which can then downregulate the transcription factor. For this reason, negative feedback loops are self-limiting (Herranz and Cohen, 2010). On the other hand, feed-forward loops can involve a transcription factor upregulating a subset of miRs, with both the transcription factor and miRs able to regulate the same target gene (Herranz and Cohen, 2010). These feed-forward regulatory loop systems deliver more sensitive and robust regulation of target genes, highlighting the importance of miR and transcription factor interaction in functional roles (Re et al., 2009). There is a growing awareness that miR-transcription factor combinations have important roles in regulating growth signals and DNA damage responses, and miRs are therefore frequently disrupted in cancer (Zhang et al., 2006).

In the T cell compartment, conditional gene targeting of either Dicer or Drosha, critical components of microRNA maturation, has highlighted the importance of miRs in T regulatory cell biology. In mice, gene targeting of either Dicer or Drosha in Treg cells results in a severe fatal lymphoproliferative autoimmune disease that is virtually identical to that observed following *FOXP3* deletion (Liston et al., 2008, Zhou et al., 2008). Since then, genome-wide FOXP3 ChIP and RNA profiling studies (Fontenot et al., 2003, Marson et al., 2007, Sadlon et al., 2010) have indicated that FOXP3 can directly regulate a number of miRs, and that the gene expression changes brought about by these FOXP3-regulated miRs play an important role in maintaining the Treg cell phenotype. For example, miR-155 and miR-146a have both been implicated in Treg maintenance, with mice deficient for miR-155, which is highly expressed in Treg cells, having significantly reduced Treg

numbers (Kohlhaas et al., 2009, Lu et al., 2009). MiR-146a deficiency in Treg cells results in reduced immunological tolerance, likely due to decreased suppression of signal transducer and activator of transcription 1 (Stat1), which is required at low levels for the maintenance of a Treg phenotype (Lu et al., 2010a). Whether FOXP3 also uses microRNAs to regulate expression of genes in epithelial cells has not yet been examined.

1.4.2 MicroRNAs in breast cancer

Expression profiling of breast cancer samples has demonstrated clear differences in miR expression between normal and primary tumour tissues, identifying both upregulated and downregulated miRs (Iorio et al., 2005). Therefore there is the potential for miRs to act as biomarkers for cancer diagnosis and pathogenesis (Castaneda et al., 2011). Altered expression of miRs in human breast cancer was first identified in 2005, with miR-encoding genes often located at sites of loss of heterozygosity, amplification, fragile sites and at other regions that are commonly disrupted in cancers (Iorio et al., 2005, Selcuklu et al., 2009, Zhang et al., 2006). In breast cancer, approximately 73% of miR genes are located in regions that have DNA copy number abnormality. This suggests that the loss or gain of genomic regions containing miRs is not a random event, and as such miRs may strongly participate in the cause of malignancy (Calin et al., 2004, Castaneda et al., 2011). MiR microarray analyses suggest that miR expression profiles have the potential to be used as biomarkers, and may prove to be powerful diagnostic tools for the detection and treatment of human cancers. For example, miR-21 is consistently overexpressed in early breast cancers and has been shown to progressively increase in expression levels from a benign to a malignant pathology (Castaneda et al., 2011). Some progress has already been made using microRNAs as diagnostic and therapeutic tools. For example, in a blinded study, a qRT-PCR diagnostic assay measuring the expression levels of 48 microRNAs was able to

successfully classify the tissue of origin of 57 metastatic brain tissues (Mueller et al., 2011). MicroRNAs have also been used for the classification of lung cancers, with more than 90% of 451 lung cancer samples successfully subtyped based on miR expression (Gilad et al., 2012). The use of miRs as therapeutic targets is still in the early stages of development, but they have been shown to be effective in animal models. For example, one study by Takeshita *et al.* demonstrated that injection of a chemically modified miR-16 precursor (a tumour suppressor miR downregulated in prostate cancer) into the tail vein of a murine model of bone-metastatic prostate cancer significantly reduced growth of the metastases compared with mice treated with a scrambled mimic (Takeshita et al., 2010). Taken together, current evidence suggests that miRs have a promising potential as diagnostic and therapeutic tools, however it is evident that further, extensive investigation into this field is required.

There are two categories of miRs in cancer: tumour suppressor miRs, which have the ability to inhibit tumourigenesis through repression of oncogenes and oncogenic miRs (also known as oncomiRs), which target tumour suppressor genes for downregulation (Zhang et al., 2007). One example of a miR that is classed as a tumour suppressor miR in breast cancer is let-7. This miR is under-expressed in many human cancers. Treatment of breast cancer cells and mouse models of breast cancer with let-7 results in reduced cell proliferation, reduced mammosphere formation and increased cell differentiation (Yu et al., 2007, Barh et al., 2010). It has also been shown to block a number of targets involved in key cancer pathways, linking let-7 to the reduction of angiogenesis, growth and metastasis in breast cancer (Barh et al., 2010). On the other hand, miR-21 is an example of an oncogenic miR, which was first identified due to its role in preventing apoptosis in glioblastoma (Chan et al., 2005). Since then, miR-21 overexpression has been identified in

many human cancers, including breast, lung, stomach and prostate cancers (Gao et al., 2012, Ribas and Lupold, 2010, Fu et al., 2011, Yan et al., 2008, Iorio et al., 2005). Upregulation of miR-21 has also been correlated with decreased sensitivity to anti-cancer agents (Pan et al., 2011). Further studies suggest roles for miR-21 in tumour cell proliferation and cell invasion in addition to its anti-apoptotic role (Ribas and Lupold, 2010, Pan et al., 2011, Zhu et al., 2008).

As is the case with many genes in human cancers, miRs are often difficult to classify as either 'oncomiRs' or 'tumour suppressor miRs', as a number of miRs can display characteristics of both. The activity of miRs can alter in a tissue, depending on the changes in the cell transcriptome, tumour stage, microenvironment and tumour activity (for example if they are involved in growth, migration or chemotherapy resistance) (Xiang et al., 2011, Olson et al., 2009). MiR-155 is an example of this, as it appears to have differing roles in breast cancer depending on the stage of cancer progression. This particular miR is significantly overexpressed in a large proportion of human breast cancers, with one study reporting overexpression in 91% of breast cancer samples (Mattiske et al., 2012). However, increased miR-155 expression has also been shown to prevent tumour dissemination and epithelial-to-mesenchymal transition in the mammary fat pads of mice, thus suggesting a role as a tumour suppressor. However once the cancer has metastasised to the lung, overexpression of miR-155 is required for the formation of macroscopic tumours, suggesting a role as an oncogene (Xiang et al., 2011). Other examples of miRs with potential oncogenic and tumour suppressive roles include the miR-200 family (Gregory et al., 2011, Bracken et al., 2008), miR-126 (Tavazoie et al., 2008, Fish et al., 2008) and miR 483 (Olson et al., 2009).

It is clear that the molecular mechanisms responsible for the regulation of oncogenes and tumour suppressors are complex and tightly controlled in healthy tissues, and that there are many points where loss of regulation will result in the promotion of cancer progression and metastasis. With recent developments in experimental technique and gene expression technology, it is now feasible to model these processes in healthy and disease cells while also gaining insight into the expression profiles of individual cancers. This will not only further understanding of cancer development, but may also allow for the identification of novel biomarkers and targets for therapy.

1.5 Summary

Breast cancer is a highly heterogeneous disease that affects a significant proportion of women. Although some critical genes involved in breast cancer have been uncovered and are used for targeted therapeutic treatment, further study into novel breast cancer genes is required. Expression of the Treg cell master regulator gene *FOXP3* has now also been identified in normal breast epithelium, but is lost or downregulated in a large proportion of breast cancers. FOXP3 is believed to play a tumour suppressive role in the breast, where it has been confirmed to downregulate expression of a number of well-established oncogenes, while also upregulating the expression of tumour suppressor genes. FOXP3 ChIP-on-chip studies in human Treg cells have identified a number of FOXP3-regulated genes, including those involved in such processes as apoptosis, growth and proliferation. Interestingly, a number of small, non-coding microRNAs were also identified as potential FOXP3 targets, which is of significance given their known deregulation in human disease, including cancers. Therefore a detailed investigation of FOXP3-mediated regulation of microRNAs in breast epithelia is required.

1.6 Hypothesis and Specific Aims

The general hypothesis of this thesis is that FOXP3 functions as a tumour suppressor in breast epithelia, in part through the regulation of specific microRNAs.

The specific aims of this PhD are:

1. To determine a direct relationship between FOXP3 and microRNAs

2. To confirm that FOXP3 and FOXP3-regulated microRNAs are involved in regulating the phenotype of breast epithelial cells

3. To confirm a causal relationship between FOXP3 and *SATB1* expression levels

4. To determine biological outcomes of *FOXP3*, miR and *SATB1* manipulation

5. To model *FOXP3* induction in breast epithelial cells and to confirm a link with the p53 DNA damage pathway

CHAPTER 2: MATERIALS & METHODS

2.1 Materials

2.1.1 Primers

Table 2.1 ***Primers used for PCR and Cloning***

	Forward (5'-3')	Reverse (5'-3')
SATB1 RT	ACAGGTGCAAAAATGCAGGGA	GCGTTTTCATAATGTTCCACCAC
RPL13a RT	CGAGGTTGGCTGGAAGTACC	CTTCTCGGCCTGTTTCCGTAG
FOXP3 RT	GTCTGGGCTCATAGGCACATT	ATGGCGTTCTGTGGAAGGC
EGFR RT	GCGTTCGGCACGGTGTATAA	GGCTTTCGGAGATGTTGCTTC
RAF-1 RT	GCACTGTAGCACCAAAGTACC	CTGGGACTCCACTATCACCAATA
PAK-1 RT	TACCAGCACTATGATTGGAGTCG	GGATCGGTAAAATCGGTCCTTCT
SKP2 RT	ACCTCCAGGAGATTCCAGACC	CCCAGGTTTGAGAGCAGTTCC
HER2 RT	GTCTCTGCCTTCTACTCTCTACC	GACAGGTCAACAGCCACATGA
P53	TGGCCATCTACAAGCAGTCAC A	GCAAATTTCCTTCCACTCGGAT
miR-7 mut SATB1 UTR	TGTTTGCAATGTGGAACCCTTTGG TTTACA	TGTAAACCAAAGGGTTCCACATT GCAAACA
miR-155 mut SATB1 UTR	GAGCCTCAAACAATCGAAATACC TTCTGTG	CACAGAAGGTATTTCGATTGTTTG AGGCTC
WT SATB1 3'UTR	CAGCCAGCTGTAACAAAATAGC	GAAGAAGAGCTGTCAGTGGAAG
SATB1	GGGGACAAGTTTGTACAAAAAAG CAGGCTTCGAAGGAGATAGAACC ATGGAGTGACTGAGTATGGATCA TTTGA	GGGGACCACTTTGTACAAGAAAG CTGGGTCTCAGTCTTTCAAATCAG TATTAAT
miR-155	GGGGACAAGTTTGTACAAAAAAG CAGGCTCATCGAAGGAGATAGAA CCATGGTGTACTGTGTCAGAATGC AAGC	GGGGACCACTTTGTACAAGAAAG CTGGGTCAGTTGCCTGAACAGAA AATCGT
miR-7-1	GGGGACAAGTTTGTACAAAAAAG CAGGCTCATCGAAGGAGATAGAA CCATGGCAGAATGCACAGTATTT GCTGC	GGGGACCACTTTGTACAAGAAAG CTGGGTCTACACCTCAAATGCAG AACACC
miR-7-2	GGGGACAAGTTTGTACAAAAAAG CAGGCTCATCGAAGGAGATAGAA CCATGGTTTCTCTTCTGCATGGTG GTTC	GGGGACCACTTTGTACAAGAAAG CTGGGTCCCCTTCAGGTAGTGGTA TGGAA
PsiCheck2 Sequencing	TGCTGAAGAACGAGCAGTAA	CGAGGTCCGAAGACTCATTT

2.1.2 Antibodies

Table 2.2 ***Antibodies used in western blots***

Specificity	Isotype	Conjugate	Source (catalogue #)
SATB1	Mouse	-	BD Biosciences, NJ USA (611182)
p53	Rabbit	-	Cell Signalling Technology Inc, MA USA (2527)
c-Raf	Rabbit	-	Cell Signalling Technology Inc, MA USA (9422)
α-tubulin	Rabbit	-	Rockland Inc, PA USA (600-401-880)
FOXP3	Goat	-	Abcam, Cambridge UK (ab2481)
Rabbit	Goat	HRP	Thermo Scientific, MA USA (32460)
Mouse	Goat	HRP	Thermo Scientific, MA USA (32430)
Goat	Mouse	HRP	Thermo Scientific, MA USA (31400)

2.2 Methods

2.2.1 Cell line culture

BT549 cells (ATCC, VA USA) were cultured in RPMI (Thermo Scientific, Waltham USA) supplemented with 10% FBS (Sigma-Aldrich, MI USA) and 1% Penicillin/streptomycin solution (Sigma-Aldrich, MI USA). MDA-MB-231 cells (ATCC, VA USA) and HEK 293T cells (ATCC, VA USA) were cultured in Dulbecco's modified Eagle Medium (DMEM; Thermo Scientific MA USA) supplemented with 10% FBS and 1% Pen/Strep. MCF10a cells (ATCC, VA USA) were cultured in medium comprising of DMEM, Hams F12, 5% Horse serum, 1% Pen/Strep, Cholera toxin, human insulin, hydrocortisone and Epidermal Growth Factor. Human Mammary Epithelial cells (HMEC, Lonza, Switzerland) were maintained in the recommended Basal medium supplemented with the HMEC Bulletkit (Lonza, Switzerland). All cell lines were maintained at 37^{o}C and 5% CO_2 in a humidified incubator.

2.2.2 Cryogenic storage of cell lines

Cell lines were pelleted by centrifugation at 300g for 5 minutes at room temperature in a sterile 50mL falcon tube and resuspended at a concentration of 2x10^{6}cells/mL in chilled

freezing medium (90% FCS+10% DMSO). 1mL of the suspension was added to 1.5mL cryovials, and placed into a Mr Frosty (Nalgene, Denmark) filled with isopropanol. The Mr Frosty containing the cells was then transferred to a -70^{o}C freezer for 12 hours, after which the cryovials were transferred to liquid nitrogen for long-term storage.

2.2.3 Thawing of cryogenically frozen cell lines

Cryovials were transferred from liquid nitrogen to a 37^{o}C water bath, and were thawed with gentle shaking in the water until the contents were partially thawed. Cryovials were wiped with 70% ethanol and warm complete medium (as described in 2.2.1) was added dropwise to the cell suspension and the cell/medium mix was transferred to a sterile 10mL tube containing pre-warmed medium. Cells were centrifuged (300g for 5 minutes at RT) and washed once, before centrifuging again and resuspending in 10mL of pre-warmed complete medium. Cells were then plated in a T75 flask, and incubated at 37^{o}C/5% CO_2 overnight, after which the medium was replaced with fresh pre-warmed medium and incubated again.

2.2.4 Construction of transfer vectors

SATB1 and microRNA sequences were cloned into lentivirus plasmid constructs using Gateway technology (Invitrogen, CA USA). Primers for these coding sequences were designed to incorporate ‘att’ sites, and PCR was performed to amplify the sequences using the KAPA HiFi DNA polymerase (Kapa Biosystems, MA USA). Following the PCR reaction, agarose gel electrophoresis was performed to confirm the size and purity of the amplified product. PCR products were then cloned into the pDONR-107 entry vector using the Gateway BP clonase II enzyme mix (Invitrogen, CA USA) according to the manufacturer’s instructions. BP reactions were transformed into DH5α competent cells (50

μL) (Invitrogen, CA USA), and recombinant clones selected for on Luria broth agar plates supplemented with Kanamycin. DNA from individual clones was isolated from overnight (2 mL) cultures using a QuickLyse Miniprep Kit (Qiagen, Germany). Restriction enzyme digests were performed on the miniprep reactions to confirm that the correct sequence was cloned into the donor vector. Gateway LR Clonase II enzyme mix (Invitrogen, CA USA) was then used to transfer the desired sequence from the donor vector into the destination transfer vector. LR reactions were set up according to the manufacturer's protocol. The reaction mixes were transformed into DH5α cells, and recombinants selected for on ampicillin plates. Plasmid DNA was isolated by QuickLyse Miniprep Kit (Qiagen, Germany) as described below. Recombinant clones were identified by restriction digestion and confirmed by sequence analysis (2.2.15).

2.2.5 DNA purification

DNA for cloning and sequencing was purified from bacterial cultures using the QuickLyse Miniprep Kit (Qiagen, Germany) or with the Nucleobond Xtra Midi kit (Machery-Nagel, Germany) for transfection of mammalian cells as per the manufacturers' protocols. DNA yield and purity was quantified using a NanoDrop spectrophotometer (Thermo Scientific, DE USA), with samples stored at -20^{o}C until use.

2.2.6 Lentivirus constructs

The lentivirus construct pLVEIG-*FOXP3* described previously (Brown et al 2010) encodes full length human *FOXP3* transcribed from the Elongation factor 1alpha (EF1α) promoter, whilst enhanced green fluorescence protein (eGFP) is expressed from an internal ribosome entry site (IRES). The production and transduction of lentiviral vectors is described in further detail in section 3.3.1. Briefly, *FOXP3* and *GFP*-expressing lentiviral preparations

were generated as previously described (Barry et al., 2001). HEK 293T cells were transfected with lentiviral transfer vector and packaging vectors using Lipofectamine 2000 transfection reagent (Invitrogen, CA USA). A total of 30 μg of DNA was used, consisting of 12.5 μg transfer vector, 7.5 μg gag/pol, 6.25 μg Rev and 3.75 μg VSV-G. Virus was harvested 72 hours post transfection and viral titres were determined. Breast cancer cell lines were transduced with lentivirus at a multiplicity of infection (MOI) of 2, and sorted for GFP on day 4. Sorted cells were cultured and used for further experiments.

2.2.7 Concentration of lentiviral supernatants

Virus supernatant was concentrated by centrifugation (90 mins at 20,000g) in a swing-out rotor (SW 32 TI Rotor, Beckman Coulter, CA USA) using an Optima Ultracentrifuge (Beckman Coulter, CA USA). Supernatant was removed and the viral pellet resuspended in 1 mL of DMEM medium containing 10% FCS and 1% Penicillin/Streptomycin (Pen/Strep). The concentration of the resulting viral resuspension was subsequently tested as described in Section 3.3.1.

2.2.8 Cell proliferation assay

The proliferative capacity of cells was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, WI USA). This kit measures the production of the coloured insoluble metabolite Formazan from the reduction of the substrate MTS tetrazolium within cells. The quantity of Formazan produced is relative to the number of living cells. To measure viability and proliferation of cell lines, cells were plated at 1 x 10^4 cells/well in 100 μL of the appropriate medium in 5 clear 96 well flat-bottomed plates. Cells were plated in triplicate and the proliferation assay performed over a 5 day period with separate plates assayed for each day. To measure Formazan production,

CellTiter 96 AQueous One Solution (20 μL) was added to triplicate wells, and the plates were incubated at 37°C/CO_2 for 30 minutes. Absorbance was measured in a plate reader at 490nm. This was repeated for each remaining day of the assay.

2.2.9 Whole cell lysate extraction

Cells growing in a flask were washed with cold PBS before being kept on ice. 500 μL of modified RIPA lysis buffer (1% Np-40, 0.25% NaDeoxycholate, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 50 mM Tris 7.7, 1X complete protease inhibitor cocktail (Roche Applied Science, Germany), 1X Pefablock (Roche Applied Science, Germany)) was then added to the flask. Cells were harvested using a rubber cell scraper, and transferred to a 1.5 mL eppendorf tube before being homogenised by drawing up and down (5x) using a 1 mL syringe fitted with a 20 gauge Precision guide needle (BD Bisociences, CA USA). The samples were then placed on a rocker at 4°C for 15 minutes, before insoluble material was pelleted in a benchtop centrifuge (16, 000g for 10 minutes at 4°C). Aliquots (100 μL) of the lysate were transferred to 1.5 mL eppendorf tubes and stored at -70°C until use. Protein concentrations in the sample were determined by the Dc Protein Assay (Bio-Rad, CA USA) as described in section 2.2.11.

2.2.10 Nuclear and Cytoplasmic Extraction

Nuclear and Cytoplasmic protein fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, IL USA). Cells were washed with ice-cold PBS before being harvested by trypsinisation and cells pelleted by centrifugation (300g for 5 minutes). The supernatant was aspirated and the packed cell estimated before the addition of 10x the packed cell volume of ice-cold CER-I reagent. After vigorous vortexing for 15 seconds, the tube was incubated on ice for 10 minutes. Ice-

cold CER-II (1/20th total volume) was then added, and the tube vortexed again for 5 seconds and incubated on ice for 1 minute, before the sample was vortexed again for 5 seconds and the nucleii pelleted by centrifugation (16, 000g for 5 minutes at 4^{o}C). The supernatant containing the cytoplasmic extract was transferred to a chilled tube, and stored at -70^{o}C. The pellet containing the nucleii was then resuspended in ice-cold NER solution (50 μL) and vortexed at top speed for 15 seconds. The sample was then returned to ice and vortexed every 10 minutes over a 40 minute timeframe. After centrifugation in a microcentrifuge (16,000g for 10 minutes at 4^{o}C), the supernatant containing the nuclear extract was transferred to a clean pre-chilled tube. The concentrations of cytoplasmic and nuclear extracts were determined using the Dc Protein Assay method described below in Section 2.2.11.

2.2.11 Protein Assay

To determine the concentration of protein in lysate extractions, Dc protein assays (Bio-Rad, CA USA) were performed in a 96 well flat-bottomed plate according to the manufacturer's instructions. Samples were assayed in duplicate, and compared with duplicate protein standards (BSA) ranging from 0 mg/mL to 10 mg/mL. The plate was incubated at room temperature for 15 minutes, before being read on a plate reader at 690nm wavelength. Protein concentrations were determined using the slope created by the BSA standard curve absorbance values.

2.2.12 RNA extraction

Total RNA was extracted from all cell lines using the miRNeasy kit (Qiagen, Germany). Cells were harvested by trypsinisation in a sterile 10 mL tube, and medium was completely aspirated. The cells were then homogenized in 700 μL QIAzol reagent using a sterile 1mL

syringe with a 0.9x25 mm Precision guide needle. Total RNA containing small RNA species was isolated according to the miRNeasy kit instructions. Briefly, homogenised samples were incubated at room temperature for 5 minutes before the addition of 140 μL of chloroform and vigorous shaking for 15 seconds. Tubes were then incubated for a further 3 minutes, before centrifugation at 12,000g for 15 minutes at 4^{o}C. After centrifugation, the upper aqueous layer containing the RNA was transferred to a new tube containing 525 μL of ethanol, and mixed by gentle pipetting. 700 μL of the samples were then applied to miRNeasy spin columns, before centrifugation at 8,000g for 15 seconds. After discarding the flow-through, 700 μL of Buffer RWT was added to the spin column, followed by centrifugation at 8,000g for 30 seconds. This was followed by 2 washes with 500 μL Buffer RPE; the first spun at 8,000g for 15 seconds, and the second spun for 2 minutes. The column was then transferred to a new collection tube, and 50 μL of RNase-free water was added. This was then centrifuged for 1 minute at 8,000g, and the resulting RNA was stored at -70^{o}C until use.

2.2.13 Nucleic Acid Quantification

Quantitation of RNA and DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE USA). An aliquot (2 μL) of sample was loaded onto the nanodrop, and the concentration of the sample determined. Protein contamination was estimated by the ratio of absorption at 260nm/280nm. All RNA samples had a 260nm/280nm ratio of approximately 2.

2.2.14 Conversion of RNA to cDNA

RNA samples were converted to cDNA using the Quantitect Reverse Transcription kit (Qiagen, Germany) as per the kit instructions. Briefly, RNA was thawed on ice, and 1 μg

was used per 20 μL reverse transcriptase reaction. Quantitect Reverse Transcription reactions were for 15 minutes at 42°C followed by 3 minutes at 95°C. Resulting cDNA was stored at -20°C until use.

2.2.15 Sequencing

DNA products were sequenced by the Molecular Pathology gene sequencing service (Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia, Australia). Samples were optimised for sequencing using the recommended Big Dye protocol. Sequence Big Dye Buffer (Applied Biosystems, CA USA), appropriate primer, Big Dye enzyme (Applied Biosystems, CA USA) and water were added to the DNA sample. PCR conditions consisted of:

94°C	5 mins	
96°C	10 seconds	repeat 30x
50°C	5 seconds	
60°C	4 minutes	
4°C	hold	

Sequencing products were precipitated with isopropanol. Briefly, 75% isopropanol (90 μL) and glycogen (1 μL) were added to the sequencing product before incubation for 1 hour at room temperature. After incubation, DNA was pelleted by centrifugation (16,000g for 20 minutes) before removing supernatant and washing with 75% isopropanol (250 μL, centrifugation at 16,000g for 5 minutes). After aspiration, pellet was air-dried and sent for sequencing.

2.2.16 Quantitative Real Time PCR

Semi-Quantitative real-time RT-PCR was performed using the KAPA SYBR Fast Universal qPCR 2X master mix kit (Kapa Biosystems, MA USA). Samples were assayed in triplicate. Briefly, 10uL reactions consisted of the appropriate primer pair (0.4 μL of 10 mM stock), cDNA (10 ng), and 1X SYBR Fast mastermix. Semi-quantitative real-time RT-PCR was performed on a Corbett real time PCR machine (Rotorgene 6000, Corbett Life Sciences, CA USA). Results from three independent experiments were analysed using Rotor-Gene 6000 software and normalised to the expression of reference transcript ribosomal protein L13a (*RPL13a*). The primers used in this study are listed in Section 2.1.1 (Table 2.1).

2.2.17 Real Time PCR Calculations

Real time RT-PCR results were calculated from the Ct values for the target gene and the control *RPL13a* as a housekeeping gene. Ct values were determined for each sample using the Rotorgene 6000 software. The amplification efficiency of each primer pair was determined with a serial dilution series using the Q-gene software package (Muller et al., 2002, Simon, 2003). The mean normalised expression level was determined using Q-gene according to equation 3 in Muller *et al.* 2002 (Muller et al., 2002).

2.2.18 MicroRNA RT-PCR

Target-specific microRNA reverse transcription was performed using the Taqman microRNA Reverse Transcription kit (Applied Biosystems, CA USA) and Taqman microRNA assay probes for the appropriate miRs (Applied Biosystems, for miR-24, -7, -19b and -155). Detection of the mature microRNA forms were performed using specific Taqman microRNA assays in the presence of Taqman Universal PCR Mastermix (Applied

Biosystems, CA USA). Assays were performed in triplicate on a Corbett Real-time machine (Rotorgene 6000, Corbett Life Sciences, CA USA) with miR-24 used as a reference miR for normalisation. Results from three independent assays were analysed using Rotor-Gene 6000 software.

2.2.19 Western Blot and Immunodetection

Western blots were performed as per the BD Biosciences protocol for western blotting (http://www.bdbiosciences.com/support/resources/protocols/monoclonal_anti.jsp). Briefly, beta-mercaptoethanol (1.8%) and 2X SDS loading buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue) was added to 100 μg of total protein extracted from cell lines, and the sample incubated at 94^{o}C for 5 minutes prior to 10% SDS-PAGE. Electrophoresis was carried out using Mini-PROTEAN tetra cell gel tanks (Bio-Rad, CA USA, 200V for 30 minutes). Proteins were transferred from the gel onto a nitrocellulose membrane using wet transfer Towbin buffer (25 mM Tris, 192 mM Glycine, 10% Methanol) to in a Mini-PROTEAN tetra cell gel tank with a transfer cartridge (Bio-Rad, CA USA) at 100V for 1 hour. Nitrocellulose membranes were blocked for 1 hour in PBS/Tween (0.1%)/5% Skim milk solution at room temperature with rocking before being incubated with primary antibody (diluted according to the manufacturer's datasheet in PBS/Tween (0.1%/5% Skim milk solution overnight at 4^{o}C on a rocker. Filters were washed 5X for 5 minutes each in PBS/Tween (0.1%)/5% Skim milk solution before the membrane was incubated with the appropriate HRP-conjugated secondary antibody (Thermo Scientific, MA USA on a rocker at room temperature for 1 hour. The membrane was washed 5 times in a PBS/Tween (0.1%) solution for 5 minutes each, before the membrane was blotted dry and incubated with either West Dura or West Femto Chemiluminescent detection solution (0.1 mL/cm^2, Pierce Biotechnology, IL USA) at

room temperature for 5 minutes. Excess solution was removed from the membrane before scanning on a G:BOX iChemi imager (Syngene, Cambridge UK). Band intensities were quantitated using ImageJ 1.43 (http://rsbweb.nih.gov/ij/). To re-probe nitrocellulose membranes, filters were stripped in 1X antibody stripping solution for 15 minutes at room temperature as per instructions for Western Blot Recycling Kit (Alpha Diagnostics, TX USA), blocked for 5 minutes in 1X Blocking Buffer and subsequently re-probed with primary antibodies as described above.

2.2.20 Luciferase Constructs and Assays

Promoter reporter constructs were used to determine the FOXP3-responsiveness of FOXP3 binding regions identified in the human *SATB1* gene. FOXP3 binding regions were cloned into the pGL4.24 vector (Promega, WI USA) downstream of a minP element and upstream of a destabilised firefly luciferase. The *SATB1* promoter reporter constructs were transfected into parental, *FOXP3*-expressing or GFP control lines together with pGL4.74 Renilla luciferase construct (Promega, WI USA) using Lipofectamine 2000 according to the manufacturer's instructions. Luciferase activity was assessed 24 hours after transfection using a Dual Luciferase kit (Promega, WI USA) as per the manufacturer's instructions in a Veritas luminescent plate reader (Promega, WI USA).

To construct the *SATB1* 3'UTR reporter constructs, the 3'UTR of *SATB1* was amplified by PCR and cloned into the psiCHECK-2 vector (Promega, WI USA). HEK 293T cells or breast cancer cell lines were then simultaneously transfected with psiCHECK-2 constructs and synthetic pre-miR molecules (Ambion, TX USA) using the Lipofectamine 2000

protocol. Transfections were performed in triplicate and luciferase activity was measured 24 hours later. These protocols are described in further detail in sections 3.3.3 and 4.3.1.

2.2.21 Cell Invasion Assay Calculations

Cell invasion assays (Cultrex Basement Membrane Extract (BME) cell invasion assays (R&D Systems, MN USA) were performed as described in section 3.3.4. The averaged relative fluorescent unit (RFU) from a dilu5tion series of cells was used to generate a standard curve by linear regression of the data points. For each assay samples, the average RFU of triplicate wells were determined, and the background subtracted from the averages. The resulting RFU was then used to solve the linear equation describing the standard curve trendline to determine the number of cells present in each sample. This final value was divided by the total number of cells plated in the top chamber ($5x10^4$) to determine the percentage of cell invasion.

CHAPTER 3: FOXP3 MANIPULATION IN BREAST CANCER CELL LINES

3.1 Introduction

Forkhead box protein 3 (FOXP3) is a member of the forkhead/winged helix family of transcription factors, and can act as either a repressor or activator of transcription (Zheng et al., 2007). This occurs by binding directly to the target gene regulatory regions or indirectly via the control of other regulators such as transcription factors (Marson et al., 2007, Zheng et al., 2007). *FOXP3* is an X-linked gene, and as such only a single allele is expressed in females due to random X-inactivation (Bennett et al., 2001). FOXP3 uses its key domains, including the zinc finger, leucine zipper, forkhead and repressor domains, to achieve its roles in DNA binding, protein complex formation, repression, activation and nuclear transport (Lopes et al., 2006).

FOXP3 expression is essential for the immunosuppressive activity of T regulatory (Treg) cells, with a loss of *FOXP3* resulting in the development of the severe autoimmune diseases Scurfy in mice and IPEX in humans (Schubert et al., 2001, Ziegler, 2006, Zuo et al., 2007b, Bennett et al., 2001). *FOXP3* expression has now also been detected in non-T cell lineages, particularly in epithelial cells of the breast, prostate and lung (Tao et al., 2012, Wang et al., 2009, Zuo et al., 2007b). Recently, *FOXP3* involvement in cancer prevention was established by the demonstration that female mice heterozygous for a *Foxp3*-null mutation develop spontaneous tumours, particularly mammary tumours, at a significantly higher frequency than wildtype littermates (Zuo et al., 2007b). In all tumours the wildtype *Foxp3* allele was located on the inactive X chromosome and was not expressed in cancerous cells, while mosaic (wildtype or mutant) *Foxp3* expression was detected in cells of adjacent normal tissue (Zuo et al., 2007b). These findings indicated that tumours were exclusively derived from cells lacking wildtype *Foxp3* expression, suggesting that in normal mice, loss of *Foxp3* expression may contribute to the

development of cancer. A reduction in *FOXP3* expression or a change in FOXP3 localisation has also been observed in a significant proportion of human breast cancer samples when compared with matched normal samples (Zuo et al., 2007b, Karanikas et al., 2008). Further investigation of the consequences of *FOXP3* loss led to the identification of FOXP3 targets in breast epithelia, including the well-established oncogenes *HER2* (Zuo et al., 2007b) and *SKP2* (Zuo et al., 2007a), and the tumour suppressors *p21* (Liu et al., 2009a) and *LATS2* (Li et al., 2011b).

Given the finding that FOXP3 downregulates a number of oncogenes while upregulating tumour suppressor genes, FOXP3 is proposed to behave as a tumour suppressor in breast epithelial cells and to be involved in the prevention of cancer progression. Consistent with this, forced expression of *FOXP3* or induction of endogenous *FOXP3* in breast cancer cell lines *in vitro* leads to significant growth inhibition and promotion of apoptosis (Zuo et al., 2007a, Zuo et al., 2007b). Importantly, loss of *FOXP3* is generally associated with a poor clinical outcome (Karanikas et al., 2008, Ladoire et al., 2012, Liu and Zheng, 2007, Zuo et al., 2007a, Zuo et al., 2007b). It is highly likely given the extensive regulatory program in Treg cells (Fontenot et al., 2003, Marson et al., 2007, Sadlon et al., 2010) and MCF-7 cells (Katoh et al., 2011) that FOXP3 has many other targets in breast epithelia that are yet to be determined.

The targets of FOXP3-mediated regulation are therefore likely to be implicated in the maintenance of breast epithelial cells. At the beginning of this study, ChIP-on-chip studies in human T cells had uncovered a number of microRNAs whose expression changed in T regulatory cells compared with T helper cells (Pederson, 2007, Sadlon et al., 2010).

Regulation of miRs by FOXP3 in breast epithelia and the consequences of this regulation have not yet been investigated. MicroRNAs (miRs) have become increasingly recognised as important mediators of disease phenotypes, and biomarkers of prognosis and therapeutic response. MiRs are deregulated in many human diseases, including breast cancer, suggesting that they may identify novel markers for detection and treatment of disease (Croce, 2009, Iorio et al., 2005, Schickel et al., 2008). The extent to which they contribute to the phenotype of a cell is poorly understood, however there have been reports suggesting that miR profiles of human cancer samples trend with the immunopathological status of the tumour, and correlate with a cancerous phenotype (Bartels and Tsongalis, 2009, Calin et al., 2005, Selcuklu et al., 2009, Volinia et al., 2006, Zhang and Coukos, 2006).

Understanding the role of FOXP3 in the development of cancer and the function of normal breast epithelial cells has important implications for human health, particularly as breast cancer affects such a large number of women. This chapter describes a mechanistic model for the tumour suppressive role of FOXP3 in breast epithelial cells. Stable introduction of *FOXP3* into the BT549 and MDA-MB-231 breast cancer cell lines results in a reduction in cancerous phenotype, which it achieves in part through the upregulation of a microRNA, miR-7.

3.2 Aims and Hypothesis

The hypothesis for this chapter is that FOXP3-regulated microRNAs contribute to FOXP3 tumour suppressor activity within breast epithelial cells.

The aims for this chapter were:

1. To determine the outcome of re-introducing *FOXP3* into breast cancer cell lines

2. To confirm the regulation of microRNAs by FOXP3 in breast cancer cell lines

3. To determine the outcome of FOXP3 and FOXP3-regulated miR expression on the proliferation and invasion of breast cancer cell lines.

3.3 Materials & Methods

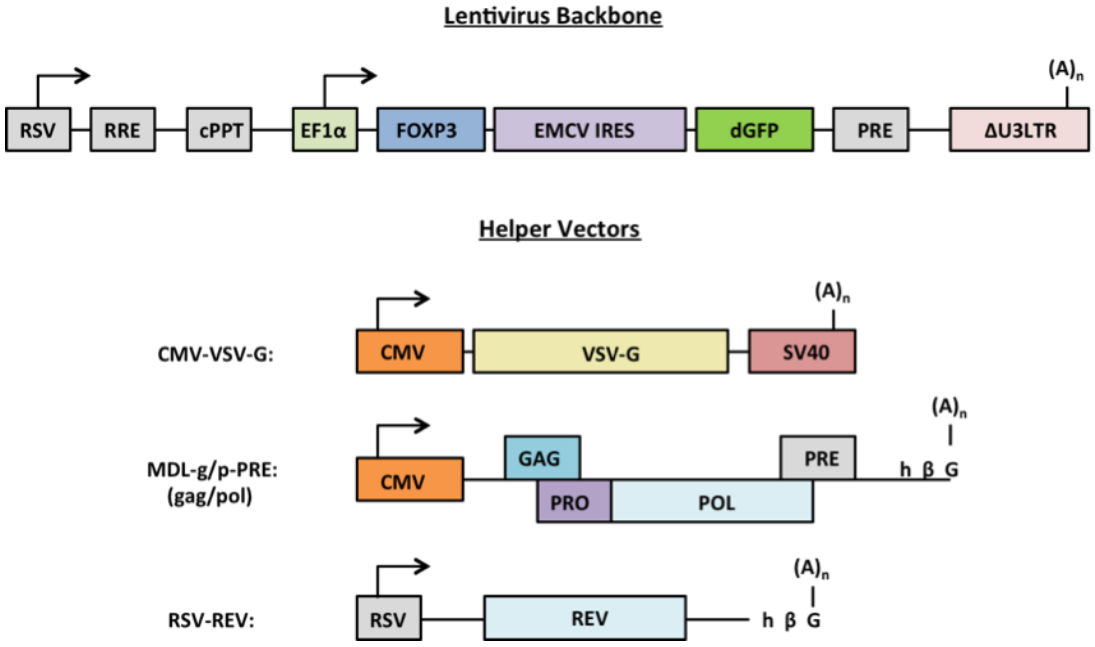
3.3.1 BT549 and MDA-MB-231 cell lines

The BT549 epithelial cell line is derived from the papillary, invasive ductal tumor of a 72 year old Caucasian woman. When isolated, the cancer had already metastasized to a number of lymph nodes. This highly aggressive estrogen receptor (ER) negative cancer cell line has a polymorphic cell composition that predominantly consists of epithelial components, but also contains some multinucleated, oversized cells (Lasfargues et al., 1978). Multinucleated cells are highly resistant to chemotherapy and have been implicated in the generation of metastatic tumours (Weihua et al., 2011). This cell line has been shown to completely lack *FOXP3* expression (Zuo et al., 2007b). The MDA-MB-231 ER-negative epithelial cell line was derived from the pleural effusions of a 51 year old Caucasian woman with metastatic breast cancer (Cailleau et al., 1974). This breast cancer cell line is highly aggressive, and like the BT549 cell line has been shown to completely lack *FOXP3* expression at both the message and protein level (Zuo et al., 2007b).

3.3.2 Lentivirus production and transduction

The lentivirus (LV) construct pLVEIG-*FOXP3* expressing full length human *FOXP3* and the control virus pLVEIG-*GFP* containing an additional *GFP* gene in place of *FOXP3* (Figure 3.1) have been described previously (Brown et al., 2010). Lentivirus preparations were generated as previously described (Barry et al., 2001). Briefly, HEK 293T ($6x10^6$/T75cm^2 flask) cells were transfected with lentiviral transfer vector and packaging vectors pCMV-VSV-G (VSV-G), pMDL-g/p-PRE (gag/pol) and pRSV-REV (REV) by transfection using Lipofectamine 2000 transfection reagent (Invitrogen, CA USA). A total of 30 μg of DNA was used, consisting of expression vectors for Gag/Pol (7.6 μg); Rev (6.4 μg); VSV-G (3.6 μg) and transfer vector containing the gene of interest (12.4 μg).

Figure 3.1 ***The vectors required for the production of the pLVEIG-FOXP3 lentivirus***



The pLVEIG-*FOXP3* lentiviral vector contains full-length *FOXP3* driven by the EF1-α promoter. An EMCV Internal Ribosome Entry Site (IRES) allows expression of the GFP reporter. LVEIG is a self-inactivating vector with a deletion in the 3'LTR. Also shown is the location of the Rev-Responsive Element (RRE), the Post-transcriptional Regulatory Element (PRE), the Rous Sarcoma Virus promoter (RSV) and the central polypurine tract (cPPT) which enhances transduction efficiency (Barry et al., 2001). Helper plasmids encode the necessary trans-elements on separate plasmids, including gag/pol and REV derived from the HIV virus, and envelope glycoproteins derived from the Vesicular Stomatitis Virus (VSV-G). (Image adapted from Tiscornia et al, *Nature Protocols* 2006 and Brown CY et al, *Human Gene Therapy* 2010).

The following day medium was replaced and virus-containing medium harvested 72 hours post transfection. Medium was passed through a 0.45 μM filter to remove cellular debris and stored at -80^{o}C prior to use.

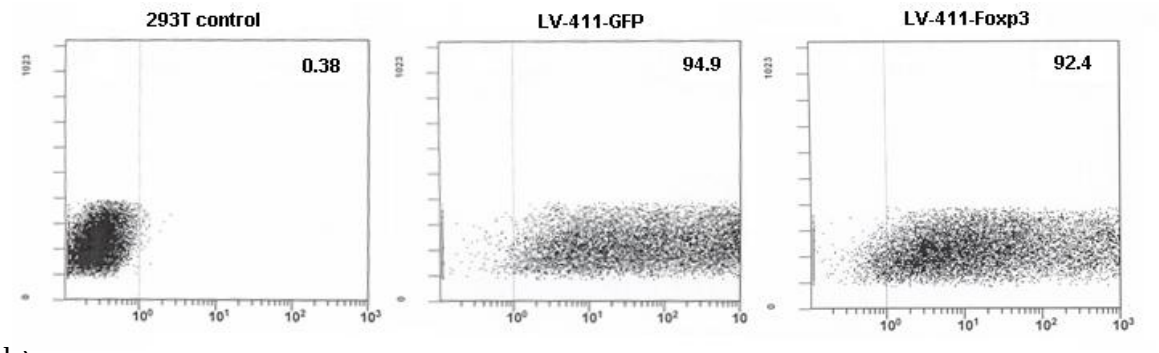
The transduction efficiencies of virus from independent packaging reactions were determined by limiting dilution transduction of HEK 293T cells. Duplicate wells of a 24 well plate containing untransfected HEK 293T cells (1x10^{5} cells/well) were infected with aliquots (50 μL) of serially diluted lentiviral supernatant (1:10, 1:50 and 1:100) in 500 μL of culture medium containing 4 μg/mL polybrene. The following day, medium was removed and replaced with fresh medium. On day 4 after virus addition, cells were harvested by trypsinisation and flow cytometric analysis was then performed to calculate the percentage of GFP-positive cells (Figure 3.2). Lentiviral titre was calculated using the equation:

$$\text{Titre} = \frac{\%\ \text{GFP positive cells} \times 1\text{x}10^{5} \times \text{dilution factor}}{\text{Volume of medium}} = \text{virus particles/mL}$$

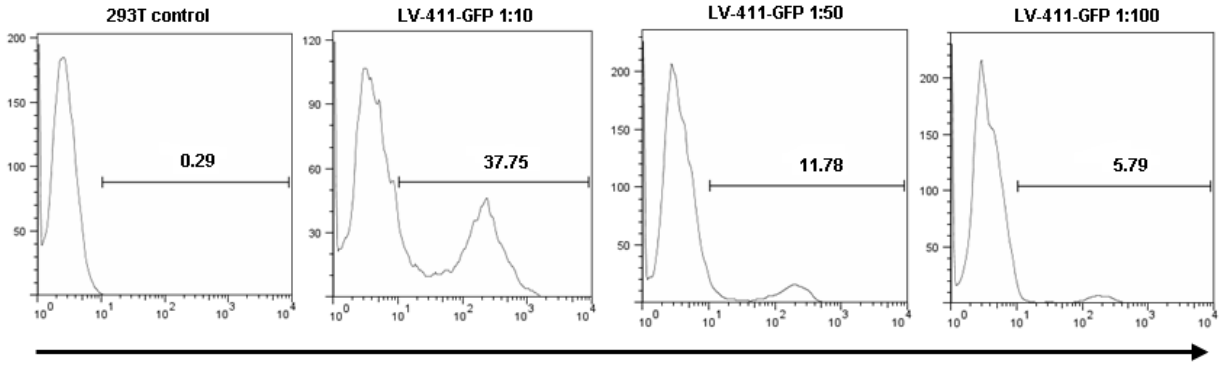
Low virus titre preparations were concentrated by centrifugation (90 mins at 20,000g, 4^{o}C) in a swing-out rotor (SW 32 TI Rotor, Beckman Coulter, CA USA) using an Optima Ultracentrifuge (Beckman Coulter, CA USA). Following centrifugation the supernatant was removed and the viral pellet resuspended in 1 mL of medium. Concentrated viral supernatants were then re-titred as above before being frozen until day of use.

Figure 3.2 ***Packaging and transduction efficiency of the pLVEIG lentivirus***

a)



b)



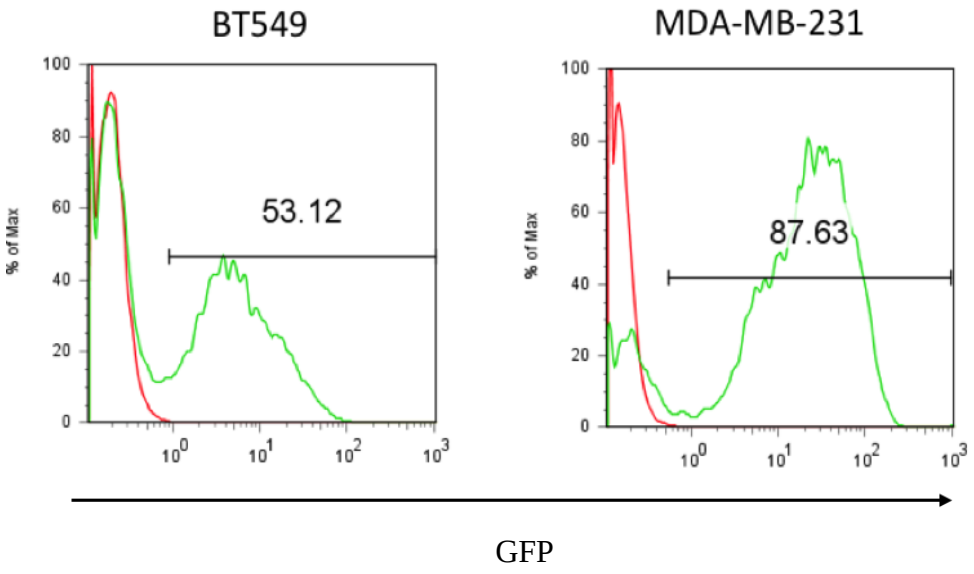
pLVEIG-*GFP* and pLVEIG-*FOXP3* lentivirus was packaged in HEK-293T cells as described in the methods. a) Transfection efficiency of the lentiviral plasmids was determined in HEK-293T cells. After harvesting virus particles from cell supernatant, cells were trypsinised and GFP fluorescence determined by flow cytometry. Fluorescence was determined relative to the un-transfected control HEK-293T line. b) The lentiviral titres of the *GFP* and *FOXP3* lentivirus supernatants were determined by treating HEK-293T with three concentrations of the supernatant, as described in the methods. On day 4, cells were trypsinised and GFP expression determined by flow cytometry. HEK-293T cells that were not transduced with a lentivirus were used as the negative control. Titre was determined from the percentage of GFP-positive cells.

The amphotrophic lentiviral particles were transduced into BT549 and MDA-MB-231 breast cancer cell lines at a multiplicity of infection (MOI) of 2. Virus was added to $1x10^6$ cells in a 6 well plate in fresh medium containing 4 μg/mL polybrene. Virus was incubated with cells overnight at $37^oC/5\%$ CO_2. The medium was then replaced with fresh medium lacking polybrene and cultured for a further 48 hours before cells were harvested and transduced (GFP^+) cells isolated by Fluorescent Activated Cell Sorting (FACS) (Figure 3.3). Sorted GFP^+ cell lines were then re-cultured and low passage number stocks cryogenically stored. Stability of viral expression was routinely tracked by flow cytometric detection of GFP. Three independent transductions of each cell line were performed.

3.3.3 Small RNA transfections of breast cancer cell lines

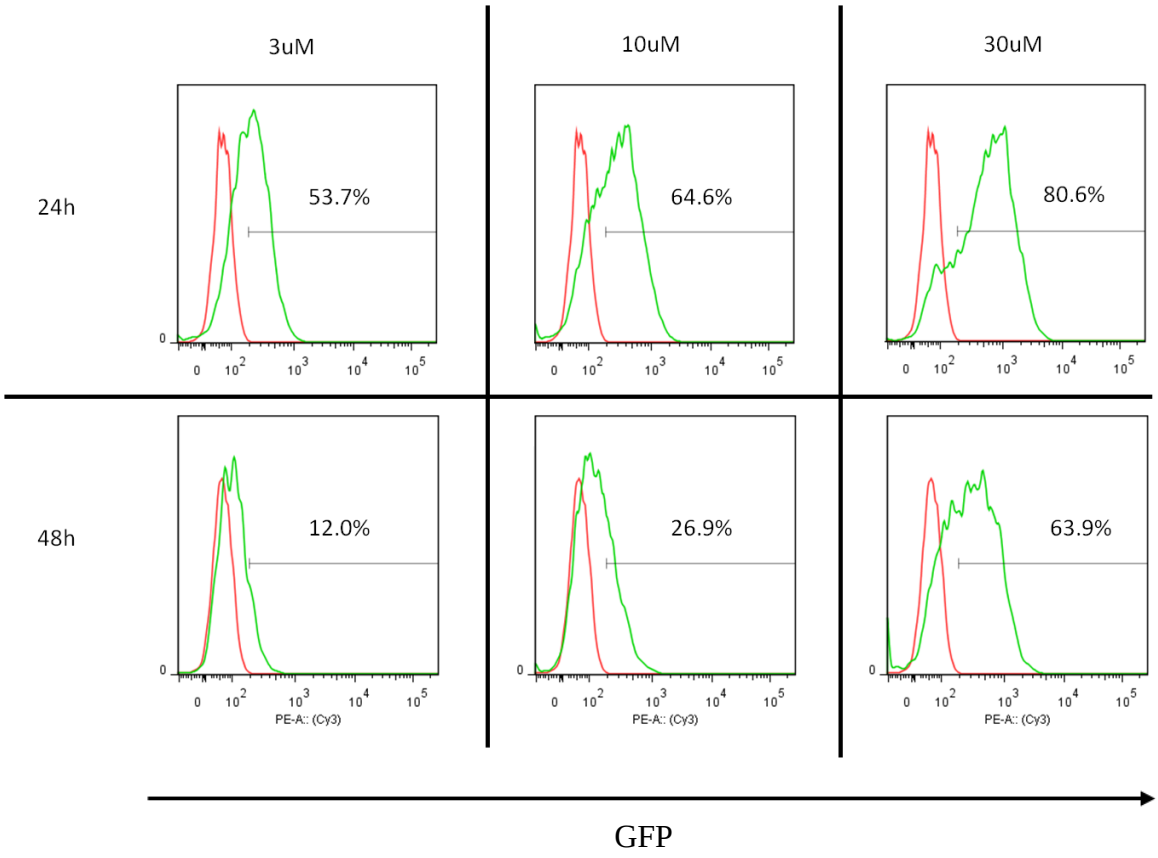
To increase specific microRNA levels, breast cancer cell lines were transfected with synthetic microRNA mimics (pre-miRs, Life Technologies, CA USA) that are processed and function as endogenous, mature miRs within the cell. BT549 and MDA-MB-231 cell lines were transfected with pre-miRs using HiPerfect transfection reagent (Qiagen, Germany) as per the manufacturer's instructions. Briefly, pre-miRs (30 μM final concentration) were diluted in serum-free medium (Optimem, Life Technologies, CA USA) and incubated with HiPerfect transfection reagent at room temperature for 5-10 minutes prior to addition to breast cancer cells ($1x10^5$ cells/well) in a 24 well plate. This concentration of pre-miR was determined to produce optimal transfection efficiencies of the breast cancer cell lines with a Cy3-labelled negative control pre-miR (Figure 3.4).

Figure 3.3 ***Transduction of breast cancer cell lines with pLVEIG-FOXP3 lentivirus***



Representative transduction of the BT549 and MDA-MB-231 breast cancer cell lines with the pLVEIG-*FOXP3* lentivirus particles. Cells were transduced at an MOI of 2 as described in the methods. The percentage of GFP positive cells was determined by flow cytometry four days post-transduction. GFP-positive breast cancer cells (green) were indicative of transduction with the pLVEIG-*FOXP3* lentivirus, while un-transduced breast cancer cell lines (red) were used as a negative control for transduction. Similar results were obtained with the pLVEIG-*GFP* virus. Data are representative of 3 independent transductions.

Figure 3.4 ***Optimisation of pre-miR transfection in the BT549 breast cancer cell line***



BT549 cell lines were transfected with a Cy3-labelled negative control pre-miR. Cells were transfected with increasing concentrations (3, 10 and 30 μM final concentration) of the Cy3-labelled control pre-miR, using a fixed volume of HiPerfect transfection reagent. Cells were collected 24 and 48 hours after transfection, and Cy3 expression analysed by flow cytometry. Cy3 positive cells (green) were compared with a parental, un-transfected cell line (red). Optimal cell transfection was observed with 30 μM of pre-miR, with maximum Cy3 expression observed 24 hours after transfection.

The day after pre-miR addition, culture medium was replaced and cells were harvested 24 hours (for RNA analysis) and 48 hours (protein analysis) post-transfection. HiPerfect transfection reagent was also used to transfect breast cancer cell lines with siRNA (final concentration of 12.5 nM) using similar conditions. The optimal concentration of siRNA was experimentally determined to produce the highest transfection efficiencies (5-15 nM final concentrations) of the breast cancer cell lines with a siGlo red-labelled negative control siRNA (Thermo Fisher Scientific, MA USA).

3.3.4 BME cell invasion assays

Cultrex Basement Membrane Extract (BME) cell invasion assays were used to characterise the invasive potential of breast cancer cell lines according to the manufacturer's instructions (R&D Systems, MN USA). Briefly, cells were incubated in normal medium containing 10% FCS overnight and then serum-starved for 24 hours in medium supplemented with 0.1% Fetal Calf Serum (FCS) prior to adding increasing concentrations of cells ($1x10^3$- $5x10^4$) to the top chamber of the assay plate. Each treatment was assayed in triplicate. The day before use, the top chamber of the invasion assay plate was coated with 50 μL of 0.1X BME coat buffer and incubated overnight at $37^oC/5\%CO_2$ in a humidified incubator. In cases where microRNA levels were manipulated in cell lines before assaying, the parental, *GFP*- and *FOXP3*-transduced breast cancer cell lines were transfected with pre-miRs as described previously (Section 3.3.2). Following an overnight incubation with the pre-miR/HiPerfect complex, the cells were serum starved in 0.1% FCS for 24 hours prior to seeding the invasion assay plates.

To calculate background signal, triplicate wells containing no cells were assayed. Medium (150 μL) either supplemented with 10% FCS or without FCS was then added to the bottom chamber and the plates incubated at 37°C/5%CO_2 for 36 hours. The top and bottom chambers were washed with wash buffer as per the manufacturer's protocol prior to addition of Calcein AM/Cell Dissociation buffer to wells in the bottom chamber. This was incubated for 1 hour at 37°C/5%CO_2, with gentle tapping at 30 minutes. The presence of invasive cells in the bottom chamber was detected by their ability to cleave the Calcein AM substrate to produce free, fluorescent Calcein. Fluorescence in the bottom plate was then read at 485nm excitation, 520nm Emission.

To calculate the number of invasive cells, a standard curve was generated for each of the breast cancer cell lines. Serial dilutions of cells (equivalent to $5x10^4$ to $1x10^3$ cells/well) in 1X cell dissociation solution were plated into triplicate wells of a 96 well plate, prior to the addition of 50 μL of Calcein AM/Cell Dissociation solution. Standard curve plates were then incubated for 1 hour at 37°C/CO_2 before being read at 485 nm excitation, 520 nm emission to obtain the relative fluorescence units (RFU). A standard curve was plotted using the average RFU for each dilution, and this was then used to calculate the number of cells that migrated in the BME invasion assay (as described in section 2.2.21).

3.4 Results

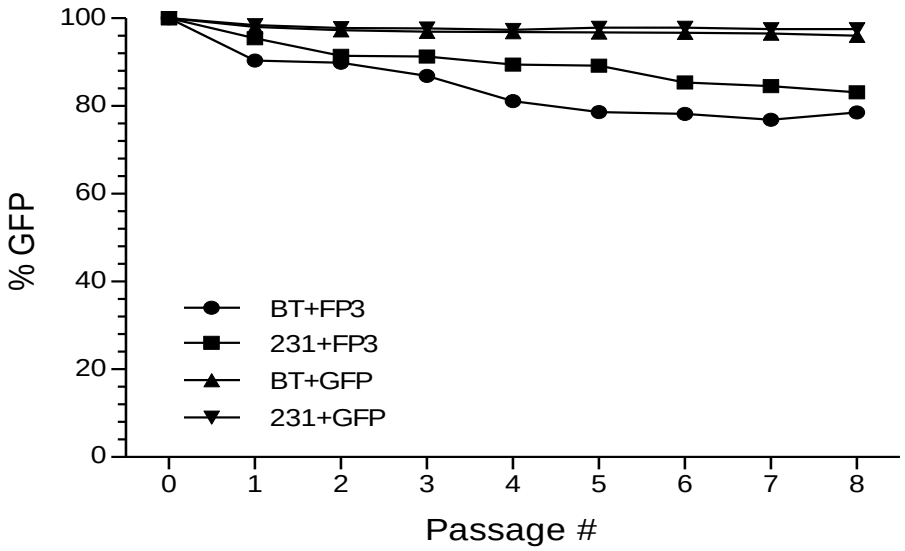
3.4.1 Stable overexpression of functional *FOXP3* in breast cancer cell lines

In order to investigate the consequences of *FOXP3* overexpression in breast cancer cell lines, lentiviral vector technology was used to create stable, long-lasting *FOXP3* expression (see Section 3.3.1). Cells that were measurably transduced with the lentivirus were isolated by Fluorescence Activated Cell Sorting based upon *GFP* co-expression from an IRES located downstream of the *FOXP3* gene. Stable *FOXP3* expression was maintained in these cell lines for up to at least 8 passages, as determined by tracking the expression of the downstream GFP marker by flow cytometric analysis (Figure 3.5) and subsequently confirmed by detection of FOXP3 mRNA and protein by qRT-PCR and western blot respectively. This stability of expression was observed in multiple independent transductions of the breast cancer cell lines. Control cells were transduced with a lentivirus containing a *GFP* gene upstream of the GFP fluorescent marker in order to remove the Gateway^{TM} cloning cassette. These cell lines also maintained expression of *GFP* over many passages.

To confirm that *FOXP3* was productively expressed in the transduced breast cancer cell lines, RNA and whole cell lysate was extracted and used for RT-PCR and western blot analysis respectively (Figure 3.6). Significantly increased FOXP3 mRNA and protein levels were observed in both the *FOXP3*-transduced BT549 and MDA-MB-231 breast cancer cell lines compared with the parental and control LV-transduced cell lines.

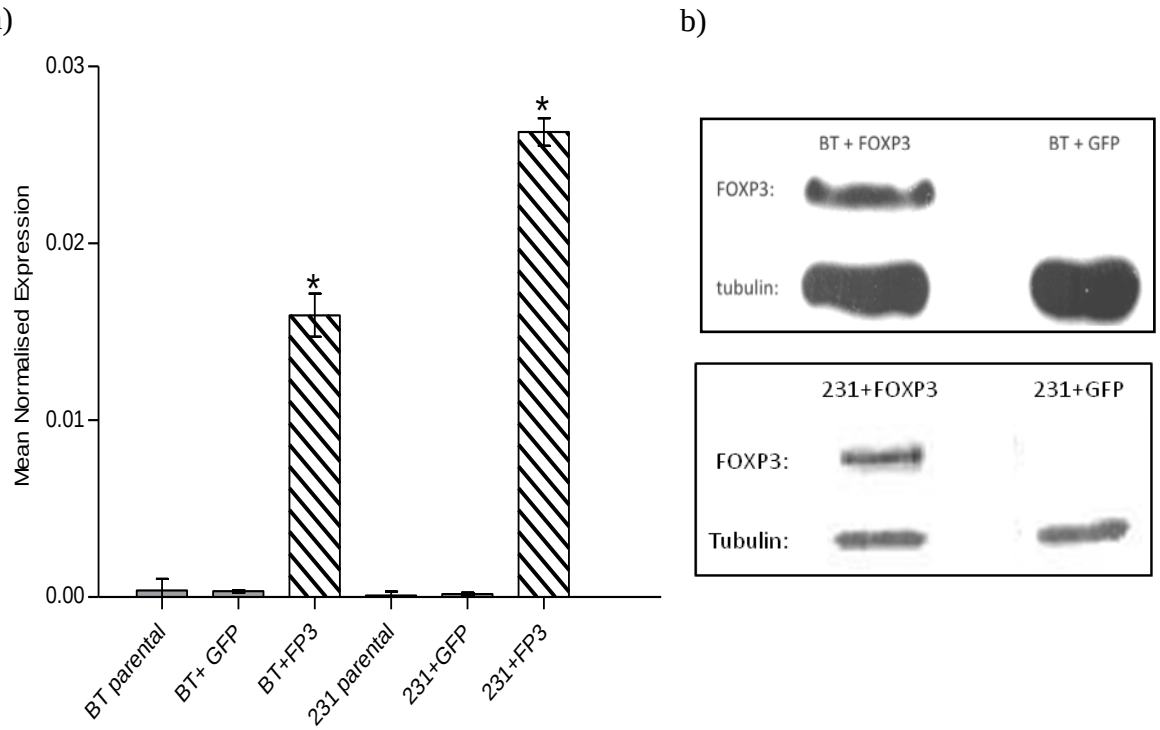
To ensure that these *FOXP3*-transduced cell lines expressed functional FOXP3 with the ability to regulate gene targets, qRT-PCR analysis was performed in the parental, GFP-

Figure 3.5 ***Stable expression of FOXP3 and GFP post-transduction***



The percentage of cells positive for GFP, expressed from an internal ribosome entry site in the pLVEIG lentivirus, was tracked for up to 8 passages post-sorting in *FOXP3* and control vector transduced cell lines by flow cytometry (n=3). Approximately 80% of BT549 and MDA-MB-231 cells transduced with the *FOXP3*-expressing lentivirus remained GFP-positive for at least 8 passages, with each passage performed approximately every 4 days.

Figure 3.6 ***FOXP3 is expressed in FOXP3-transduced breast cancer cell lines***



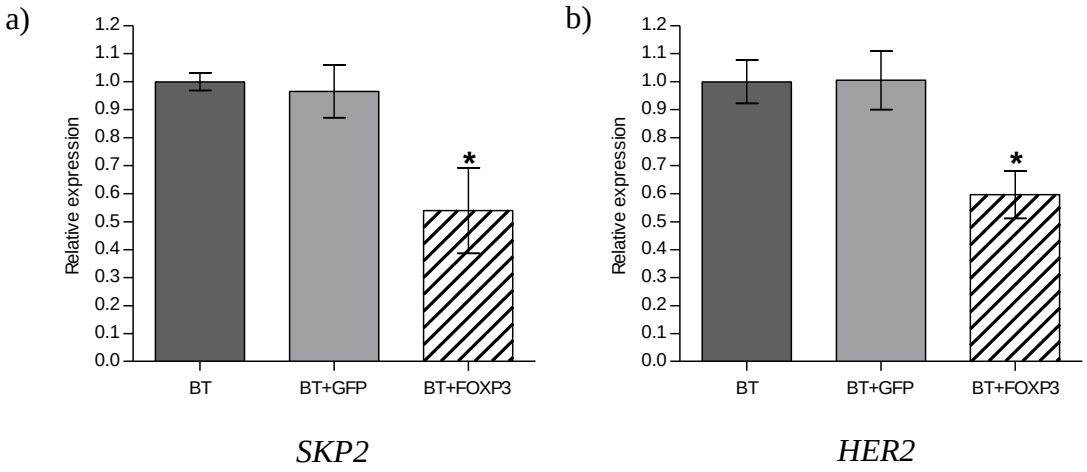
FOXP3 is expressed by *FOXP3*-transduced breast cancer cell lines at both the message and protein level. a) Total RNA extracted from BT549 (BT) and MDA-MB-231 (231) lines was used in RT-PCR experiments to determine *FOXP3* message levels. No significant changes were observed between the control GFP-transduced cell lines and the parental cell lines. n=3 independent transductions, *$p<0.0001$. b) FOXP3 protein levels as determined by western blot. Whole cell lysate extracted from BT549 (top) and MDA-MB-231 (bottom) cell lines was subjected to western blotting with a FOXP3 antibody. An α-tubulin antibody was used as a loading control. Figure is a representative of 3 independent experiments.

transduced control and *FOXP3*-transduced BT549 cell lines to determine the expression levels of two established FOXP3 targets in breast epithelial cells; *SKP2* and *HER2* (Figure 3.7). Transduction with the *FOXP3* lentivirus resulted in significantly reduced levels of both *SKP2* (~45% reduction) and *HER2* (~40% reduction), confirming that cells transduced with the *FOXP3* lentivirus are able to produce functional FOXP3 protein. No significant difference in expression levels of these two target genes were observed in the GFP-transduced control cells compared with the parental cell line, confirming the specificity of induction. These data confirm the FOXP3-dependent repression of *HER2* and *SKP2* in the BT549 cells.

3.4.2 *FOXP3* overexpression reduces proliferative potential of breast cancer cell lines

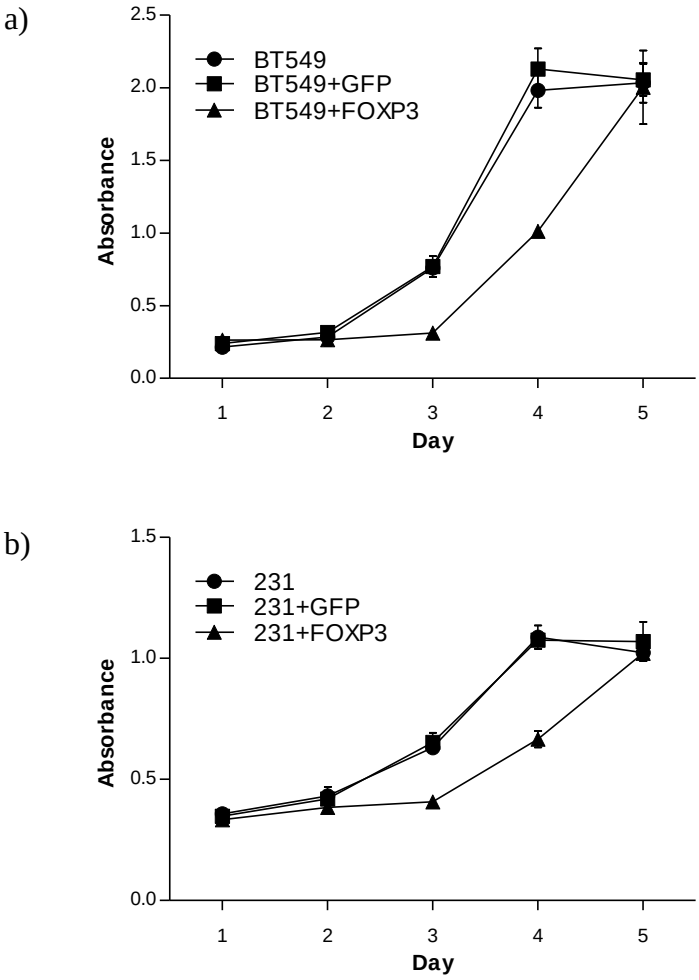
Studies by Zuo *et al.* have shown that overexpression of *FOXP3* in the ER^+, *p53* wildtype MCF-7 breast cancer cell line results in cell death (Zuo et al., 2007b). To test whether this finding was consistent in the BT549 and MDA-MB-2312 cell lines, the cell growth and viability of the *FOXP3*-expressing cell lines were compared with the control-transduced and parental cell lines over a 5 day period using the CellTitre96 AQueous One Solution kit (Promega, WI USA). Introduction of *FOXP3* into both the BT549 and the MDA-MB-231 breast cancer cell lines significantly reduced the proliferative potential of the cell compared with the parental and control *GFP*-expressing cell lines (Figure 3.8). *FOXP3*-expressing cell lines showed reduced Formazan production in the assay on days 3 and 4, although by day 5 Formazan production as measured by absorption at 490nm was similar between all three cell types. As the signal observed for the parental and GFP lines appeared to plateau at day 4 in both cell lines, it is likely that the cells had reached the maximum detection

Figure 3.7 ***FOXP3 overexpression results in knockdown of the known FOXP3 breast cancer targets SKP2 and HER2***



Relative *SKP2* (a) and *HER2* (b) mRNA levels in the parental (dark grey) and GFP-transduced (light grey) BT549 breast cancer cells compared with *FOXP3*-overexpressing (hatched) BT549 cells as determined by qRT-PCR. A significant reduction in the levels of *SKP2* and *HER2* were observed in cells transduced with *FOXP3* compared with parental and *GFP*-transduced cells (n=3, *p<0.007).

Figure 3.8 ***Proliferative activities of breast cancer cell lines***



The proliferation of BT549 (a) and MDA-MB-231 (b) cells expressing *FOXP3* was compared with parental and control *GFP*-expressing cells using the CellTiter 96 AQueous assay (Promega). Both the BT549 and MDA-MB-231 *FOXP3*-transduced cell lines had significantly reduced proliferative activity at days 3 and 4 ($p \leq 0.03$), but no significant change in proliferation was observed in the parental cell line compared with the *GFP*-transduced control cell lines. n=3 independent transductions.

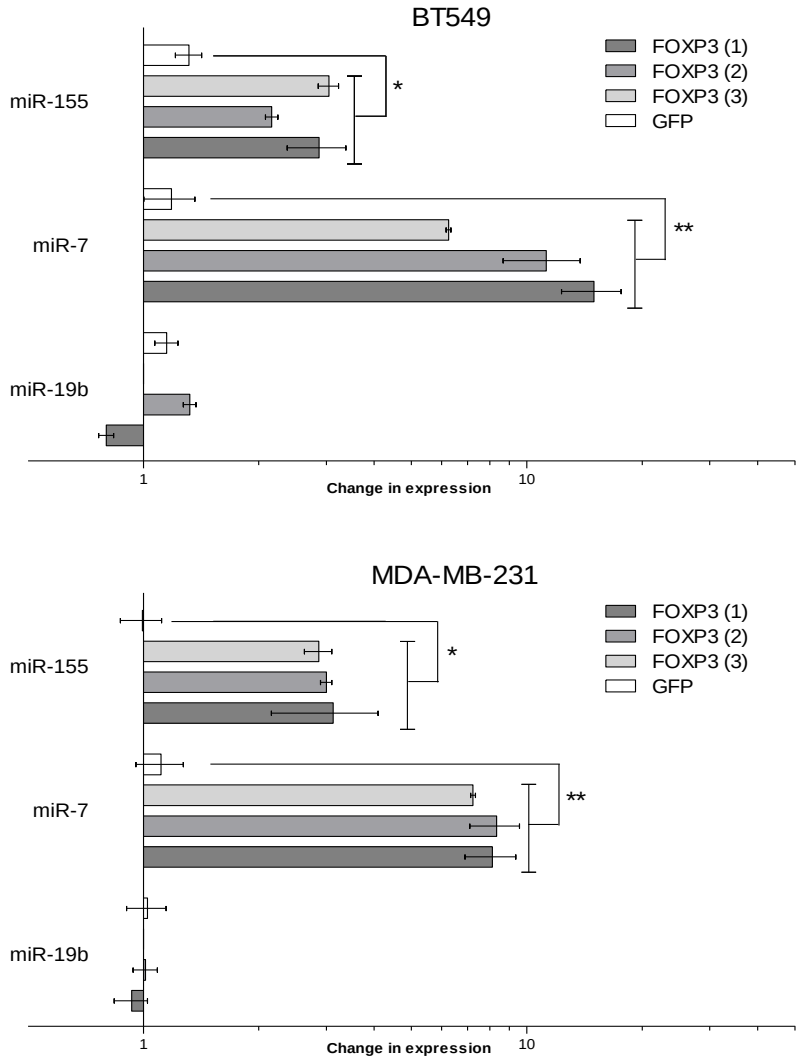
limit of the assay at this point. The reduction in absorbance measurements observed for the *FOXP3*-transduced line may indicate either a proliferative defect in cells expressing *FOXP3*, decreased plating efficiency or increased cell death. However, as similar absorption readings for *FOXP3*-expressing cells compared with the control cell lines were observed on days 1 and 2, it is unlikely that the difference on days 3 and 4 are due to decreased plating efficiency of *FOXP3*-expressing cells, indicating that *FOXP3* expression reduces cell proliferation or cell viability.

3.4.3 MiRs are induced by FOXP3 in breast cancer cell lines

Previous studies performed by the Barry group in human Treg cells indicated that a number of microRNAs are potentially regulated by FOXP3, given the presence of FOXP3 binding sites located in close proximity to the loci encoding these miRs (Sadlon et al., 2010). This work showed that FOXP3 regulates the levels of a subset of these miRs, including miR-19b, miR-7 and miR-155, and interestingly these miRs have also been implicated in breast cancer (Fang et al., 2012, Foekens et al., 2008, Xiang et al., 2011, Yan et al., 2008). This raised the question as to whether miRs form part of the *FOXP3* tumour suppressor function in breast epithelial cells. The expression of these miRs and their response to changes in *FOXP3* expression levels were therefore investigated in the BT549 breast cancer cell line.

To determine if FOXP3 is able to influence the expression levels of these miRs, miR expression was assayed in BT549 cell lines transduced with the *FOXP3* lentivirus and compared with parental and GFP control cell lines. Changes in miR levels were assayed by Taqman miR-specific RT-PCR in total RNA isolated from three independent transduction experiments (Figure 3.9). When compared with the parental and *GFP*-transduced lines, the

Figure 3.9 ***FOXP3 overexpression induces miRs -7 and -155 in BT549***

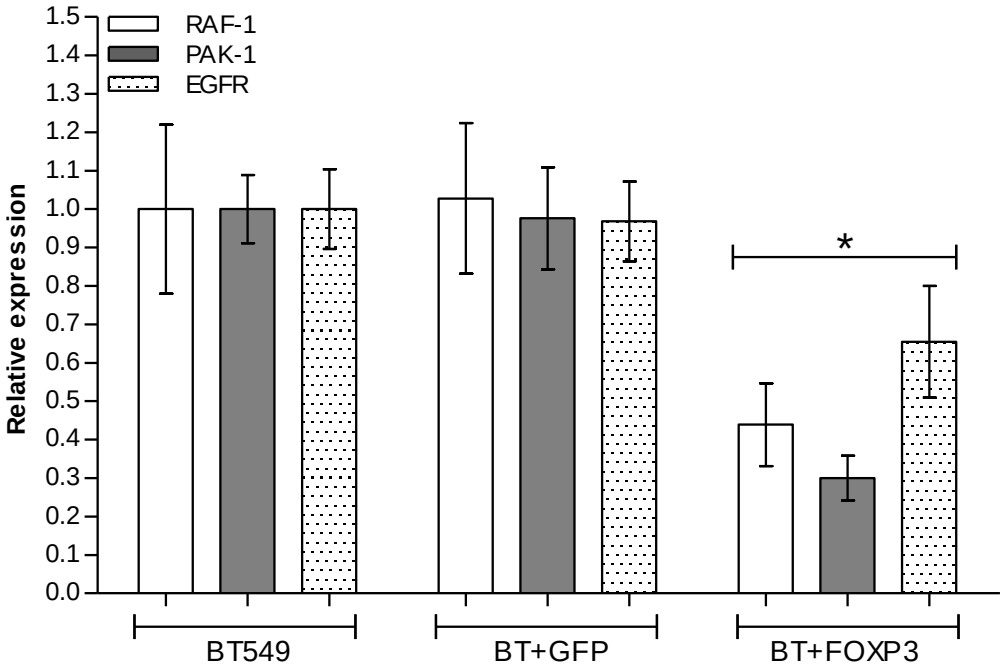


Relative levels of miR-7, miR-155 and miR-19b in three independent *FOXP3*-overexpressing pools of BT549 (a) and MDA-MB-231 (b) cells compared with parental cells. MiR levels were detected by Taqman miR-specific RT-PCR, and the fold change in expression in cells expressing *FOXP3* or *GFP* relative to parental cells was calculated. Significant upregulation of miR-7 and miR-155 was observed in *FOXP3*-expressing cells (grey bars) compared with parental and *GFP* only control cells (white bars), (n=3, *p=0.007, **p=0.0008 for miR-7 and miR-155 respectively). No change in miR-19b levels were observed in two independent transductions, and was therefore not assayed for in the third transduction.

FOXP3-transduced lines showed a significant increase in the levels of miR-7 and miR-155 (average 10-fold and 3-fold respectively, *n*=3, p=0.0008 and p=0.007 respectively). In contrast, no change in the level of another candidate miR, miR-19b, was observed. In addition, no significant changes in miR expression levels were observed in the *GFP* control lentivirus-transduced cells when compared with the parental cell line, supporting the hypothesis that the induction of these miRs occurs in a FOXP3-dependent manner. This therefore links the expression of *FOXP3* with the maintenance of endogenous miR-7 and miR-155 levels.

To determine whether the upregulation of endogenous miRs upon lentiviral overexpression of *FOXP3* is sufficient to alter miR activity in the cell, the expression of known miR-7 targets was examined (Figure 3.10). Analysis of the parental, *GFP* control and *FOXP3*-transduced BT549 cell lines by qRT-PCR indicated that the levels of the established miR-7 targets *RAF-1, PAK-1* and *EGFR* were significantly reduced in cells transduced with the *FOXP3* lentivirus (levels reduced by ~60%, ~70% and ~35% respectively) compared with the parental and *GFP* control cell lines. No significant difference in expression levels were observed in the *GFP*-transduced cell lines compared with the parental cell line, confirming that this downregulation was a FOXP3-dependent effect. These data suggest that *RAF-1*, *PAK-1* and *EGFR* levels are reduced in the presence of FOXP3, in part due to the upregulation of endogenous miR-7 levels, and thus link *FOXP3* expression with a pathway that is activated in breast cancer. The outcome of miR-155 upregulation was also investigated in the breast cancer cell lines, and is described in further detail in Section 4.4.4.

Figure 3.10 ***Validated miR-7 targets are decreased in FOXP3-transduced cells***



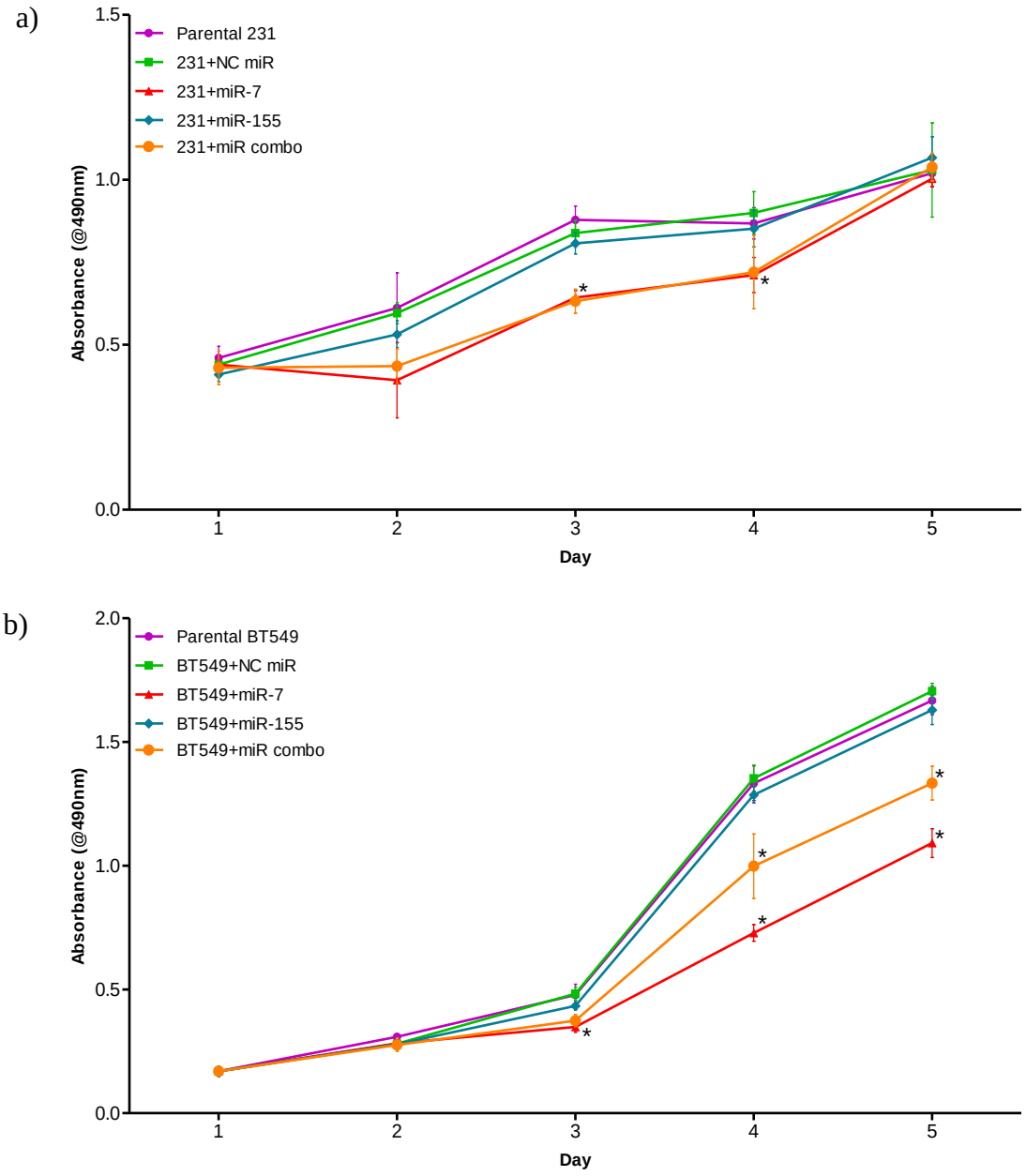
The relative mRNA levels of the validated miR-7 targets, *RAF-1* (white bars), *PAK-1* (grey bars) and EGFR (dotted bars), were determined by qRT-PCR in parental and control *GFP*-transduced BT549 cells compared with *FOXP3*-transduced cells. Total RNA was isolated from parental and transduced cell lines, converted to cDNA using the Quantitect Reverse Transcription kit (Qiagen, Germany) and qRT-PCR performed using the KAPA SYBR Fast mastermix (Kapa Biosystems, MA USA). A significant reduction in the mRNA level of these targets was observed in *FOXP3*-expressing cells, but no significant changes were observed in the control *GFP*-transduced cells. Mean relative expression from three independent experiments, *$p \leq 0.007$.

3.4.4 miR-7 and miR-155 influence the proliferative potential of breast cancer cell lines

As introduction of *FOXP3* into BT549 and MDA-MB-231 cells was shown in section 3.4.2 to reduce proliferative potential and/or cell viability, it was of interest to determine if reduced proliferation was a consequence of increased expression of the FOXP3-regulated miRs, miR-7 and miR-155. To examine this, parental BT549 and MDA-MB-231 cell lines were first transfected with pre-miR specific for miR-7 and miR-155, before their proliferative potential was examined using the CellTitre96 AQueous assay. The proliferative activity of the cell lines transiently transfected with pre-miRs were compared with parental and control pre-miR-transfected cell lines over a period of 5 days (Figure 3.11).

In both the MDA-MB-231 and BT549 cell lines, no difference in proliferative activity was observed between the parental and the control-pre-miR transfected cell lines, indicating that the transfection process did not influence proliferation. Transfection with pre-miR-155 also did not result in a significant difference in proliferative activity, suggesting that this miR does not play a role in this biological process. However, transfection with pre-miR-7 resulted in a reduction in proliferation and/or viability. Significantly reduced absorption was observed in pre-miR-7-transfected cells compared with the control cell lines on days 3, 4 and 5 in the BT549 cell line ($p<0.03$) and on days 3 and 4 in the MDA-MB-231 cell line ($p<0.03$). This significant drop in proliferative activity suggests that miR-7 may function to suppress growth and proliferation in breast epithelia, consistent with the reported tumour suppressor function in other cell types (Fang et al., 2012, Kefas et al., 2008, Webster et al., 2009). Transfection of the cell lines with a combination of both pre-miR-7 and pre-miR-155 produced either a similar reduction in absorption reading or approximately half the

Figure 3.11 ***Proliferative activity of miR-transfected breast cancer cell lines***



The proliferation of MDA-MB-231 (a) and BT549 (b) breast cancer cells transfected with 10 μM pre-miR-7 (red), pre-miR-155 (blue) or a total 10 μM combination of both pre-miRs (orange) was compared with parental (purple) and control miR-transfected (green) cells using the CellTiter 96 AQueous assay (Promega). N=3, *p<0.03.

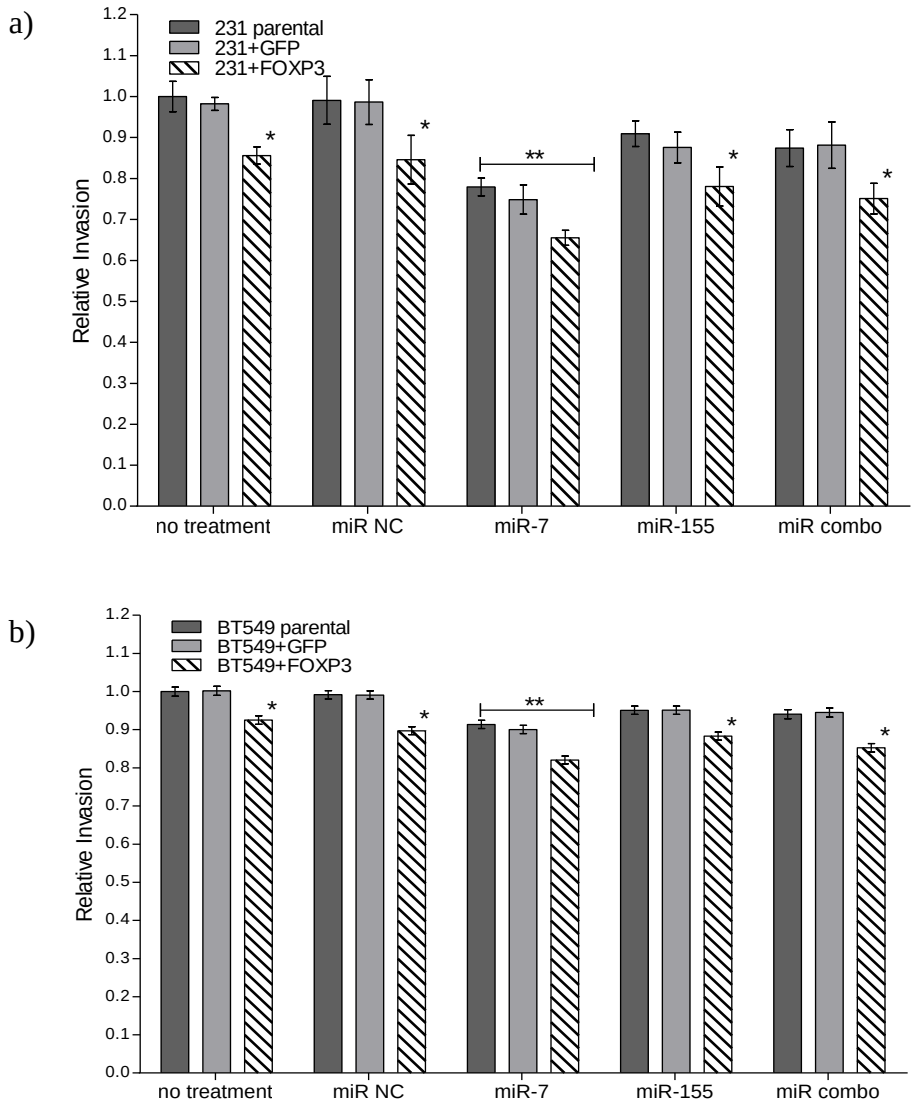
reduction observed in the miR-7 only transfected MDA-MB-231 or BT549 cell lines respectively.

3.4.5 Overexpression of *FOXP3* and miR-7 reduces invasive ability of breast cancer cell lines

To test the impact of *FOXP3* expression on one of the hallmarks of cancer, invasion, the invasive ability of breast cancer cell lines was tested using the Basement Membrane Extract (BME) cell invasion assay (described in section 3.3.2). BT549 and MDA-MB-231 cell lines were transduced with *FOXP3*, and/or transfected with pre-miRs for miR-7 or miR-155, with the resulting outcome on the invasive ability of the cells determined by calculating the percentage of cells passing through the basement membrane (Figure 3.12).

In both the MDA-MB-231 and the BT549 breast cancer cell lines, a small but significant decrease ($p<0.02$) in invasive potential was observed when *FOXP3* was expressed, with a 15% and 10% reduction in invasion observed in *FOXP3*-expressing MDA-MB-231 and BT549 cell lines respectively compared with the parental and *GFP*-transduced lines. No significant difference was observed when cells were transfected with pre-miR-155 or the non-specific control pre-miR, however a significant drop ($p<0.004$) in invasive ability resulted from transfection with the pre-miR-7 construct. This drop was observed in all cell lines, however the most significant reduction was seen when *FOXP3*-transduced cells were transfected with pre-miR-7. Treatment of the MDA-MB-231 parental and *GFP*-transduced lines with miR-7 resulted in approximately a 20% reduction in invasiveness, while transfection of *FOXP3*- transduced lines with pre-miR-7 resulted in an approximate 35% reduction. A similar result was observed in the BT549 cell line; however the drop in

Figure 3.12 ***Overexpression of FOXP3 and miR-7 results in reduced invasive ability***



Basement membrane extract (BME) cell invasion assays of parental (dark bars), *GFP*-transduced (grey bars) and *FOXP3*-transduced (hatched bars) breast cancer lines. BT549 (a) and MDA-MB-231 (b) cells transfected with 10 μM pre-miR-7, pre-miR-155 or a combination of both (5 μM each miR). A significant reduction (*$p<0.02$) in invasive ability was observed in *FOXP3*-transduced cell lines when compared with the parental and *GFP*-transduced control cell lines. A reduction in invasive ability was also seen in all cell lines when transfected with pre-miR-7 (**$p<0.004$) Larger reductions in invasive ability were seen in the MDA-MB-231 cell lines compared with the BT549 cell lines ($p<0.05$). n=4.

invasive ability was smaller than that observed in the MDA-MB-23l cell lines with an ~10% drop in *GFP*-transduced or parental lines and ~20% drop in the *FOXP3*-transduced line respectively. Transfection of BT549 cells with miR-7 alone resulted in an ~10% drop in the invasive ability of all cell lines, while in the MDA-MB-231 cell lines, an ~20% drop was observed. No significant difference was observed when cells were transfected with a combination of miR-7 and miR-155, when compared with cells transfected with miR-7 alone. Importantly, no difference in invasive ability was observed between the untreated cell lines and those treated with the pre-miR negative control, demonstrating the specificity of the response. These results suggest that FOXP3 and the FOXP3-regulated microRNA miR-7 both have the ability to influence the invasive potential of cells, resulting in reduced invasion in the breast cancer cell lines BT549 and MDA-MB-231.

3.5 Discussion

This chapter describes the stable over-expression of *FOXP3* in two breast cancer cell lines and the identification of two microRNAs, miR-7 and miR-155 that are upregulated by *FOXP3* expression. Experiments demonstrated that FOXP3 and the FOXP3-regulated miR-7, but not miR-155, can negatively regulate the growth and invasiveness of these cells. Previous studies performed by the Barry group and others have investigated the genes and pathways regulated by FOXP3 in human Treg cells, via comparison with *FOXP3*-negative T helper cells (Fontenot et al., 2003, Marson et al., 2007, Sadlon et al., 2010). At the beginning of this study, this allowed unique insight into the molecular targets of this transcription factor, some of which may also be targets for FOXP3-regulation in breast epithelia. Deregulation of these targets through the loss of FOXP3 function in breast cancer may then contribute to breast cancer progression. This chapter investigates the role of FOXP3 in regulating the phenotype of breast cancer cells, with particular focus on the BT549 and MDA-MB-231 cell lines that have been shown to completely lack *FOXP3* expression (Zuo et al., 2007b).

FOXP3 overexpression was established in these highly aggressive breast cancer cell lines using a lentiviral transduction system, with expression confirmed at both the message and protein levels (Section 3.4.1). Previous studies by Zuo *et al.* reported that introduction of *FOXP3* into the breast cancer cell line MCF-7 resulted in rapid apoptosis of the cell line (Zuo et al., 2007b, Zuo et al., 2007a). This is in contrast with observations in the BT549 and MDA-MB-231 cell lines, with overexpression of *FOXP3* only resulting in a reduction in growth. The reason for this difference is not currently known. Functional FOXP3 was produced in this study, as full-length protein was detected by western blot, and critically, repression of established FOXP3 targets, *SKP2* and *HER2* was observed (Section 3.4.1).

The differences in responsiveness to FOXP3 observed in this study compared with those reported by Zuo *et al.* could reflect differences in expression levels from a lentiviral delivered gene, particularly as a low MOI was used herein, compared with a transfected plasmid-based expression system used by Zuo *et al*. In the sorted *FOXP3*-transduced BT549 and MDA-MB-231 cell lines, no significant outgrowth of *FOXP3*-negative breast cancer cells was observed, with long-term tracking of GFP expression in FACS-sorted cell lines showing that cells maintained high percentages of GFP-positive cells over a period of 8 passages (Section 3.4.1). This suggests that *FOXP3* expression was not switched off, and therefore both breast cancer cell lines tolerated expression, a finding that was confirmed by western blot analysis for FOXP3. It may be expected that the non-transduced cell population would outgrow the FOXP3-transduced population, however this outgrowth was not observed. One possible explanation is that the gating strategy used for determining the percentage of GFP^+ cells under-reported the presence of GFP. Although lower percentages of GFP-positive cells were seen in the *FOXP3*-transduced cell lines compared with the *GFP* control-transduced lines, this did not drop below 80%, and therefore the cell population was overall considered to be *FOXP3*-tolerant. It is also important to note that the *GFP* control lentivirus contains 2 copies of *GFP*, and as such it is not unexpected that *GFP* expression levels are higher in the cells transduced with this virus. Another possible reason for the contrasting results seen in the MCF-7 cells compared with the BT549 and MDA-MB-231 cells, is that these cell lines represent different subtypes, aggressiveness and tumour locations, and as such it is possible that the different outcomes correspond to differences in the ability of the breast cancer cell lines used to respond to the transcript program normally driven by FOXP3. Consistent with this, expression profiling of these cancer cell lines showed large scale differences in gene expression (Kao et al., 2009).

Although the growth defect observed when *FOXP3* was transduced into the BT549 and MDA-MB-231 cell lines (Section 3.4.2) was small, it was consistent with previous studies also showing that FOXP3 has growth suppressing properties other breast cancer cell lines (Li et al., 2011b, Zuo et al., 2007a, Zuo et al., 2007b). These data indicate that FOXP3 tumour suppressive properties may be linked to its ability to inhibit proliferation, and as such requires further investigation.

The Treg studies performed previously by the Barry group identified a group of miRs whose expression was altered depending on *FOXP3* expression levels, with a number of miRs containing potential binding regions upstream of the miRs, including miR-7, miR-155 and miR-19b (Sadlon et al., 2010). MiR-specific RT-PCR quantitation of miR expression levels in *FOXP3*-transduced cell lines compared with parental and *GFP*-transduced cell lines revealed that endogenous levels of both miR-7 and miR-155 are upregulated upon addition of *FOXP3* (Section 3.4.3). In contrast, no change in miR-19b levels was detected, and for this reason this miR was not investigated further.

Aberrant expression of miR-7 and miR-155 have been observed in cancer, with most evidence indicating that miR-7 has tumour suppressive properties in a number of tissues including breast epithelia (Fang et al., 2012, Kefas et al., 2008, Reddy et al., 2008, Foekens et al., 2008). Although Foekens *et al.* reported reduced miR-7 levels in human breast cancer samples compared with matched normal samples (Foekens et al., 2008), recent data have suggested that miR-7 function may be dependent on the ER status of the breast cancer. Studies comparing ER^+ BC samples with ER^- BC samples suggest that miR-7 positively correlates with ER^- breast cancers (Foekens et al., 2008, Kong et al., 2012, Lyng

et al., 2012). This raises the possibility that miR-7 activity is influenced by signalling. One key tumour suppressive role for miR-7 is the suppression of epidermal growth factor receptor intracellular signalling, which it achieves through the downregulation of key components of the pathway. These include the epidermal growth factor receptor (*EGFR*) (Webster et al., 2009), a gene that is often overexpressed in epithelial tumours, p-21 activated kinase 1 (*PAK-1*) which is heavily involved in breast cancer metastasis (Fang et al., 2012) and *RAF-1,* also involved in the *EGFR* pathway. The EGFR signalling pathway is often mutated in epithelial cancers, including breast cancers, and has been shown to result in uncontrolled cell division – one of the hallmarks of cancer. In this work, upregulation of miR-7 as a result of transducing the BT549 and MDA-MB-231 cells with *FOXP3* resulted in the downregulation of *EGFR*, *RAF-1* and *PAK-1*, linking the expression of *FOXP3* with the control of the EGFR signalling pathway. Importantly, the intracellular components PAK-1 and RAF-1 are also involved in other cellular processes, with PAK-1 important for cell motility, proliferation, survival and the organisation and function of the cytoskeleton and extracellular matrix (Kichina et al., 2010), while RAF-1 is involved in proliferation, differentiation and apoptosis (Chen et al., 2001). In addition to these miR-7 targets, other known miR-7 targets include insulin receptor substrates (IRS) 1 and 2, key components of the P13K/AKT (Fang et al., 2012) and ERK/MAP kinase signalling pathways (Weng et al., 2001), and the Focal Adhesion Kinase (FAK-1), which is critical for PTK2/FAK-1 signalling (Kong et al., 2012). Together these data indicate that FOXP3, through the regulation of miR-7 expression, can influence a number of signalling pathways involved in cell motility, proliferation and survival.

There is also some controversy surrounding the role of miR-155 in human breast cancer. Multiple studies suggest that miR-155 can function as a tumour-promoting oncomiR

(Mattiske et al., 2012, Wang and Hua, 2012, Kong et al., 2010); while other studies report that it can have tumour suppressor activity (Xiang et al., 2011, Levati et al., 2009). This discrepancy in miR-155 activity could reflect the multiple different mutation and expression profiles within highly heterogeneous cancer types such as breast cancer. Alternatively, as recently shown by Xiang *et al.*, miR-155 influence on the breast cancer phenotype may depend on the stage of cancer progression and the cancer hallmark being examined. For example, miR-155 was found to play a key role in the prevention of metastasis in mouse xenograft models by reducing the levels of transcription factor 4 (*TCF4*), an important regulator of epithelial to mesenchymal transition (EMT) (Xiang et al., 2011). EMT is a key process in cancer progression, as it allows cells to move and disseminate to organs distant to the site of the primary tumour (Guttilla et al., 2012). Interestingly, this same study also reports that once breast tumour cells enter the blood stream, miR-155 is responsible for the promotion of macroscopic tumour formation at the target site of metastasis (Xiang et al., 2011), suggesting that miR-155 can also have growth-promoting properties. This may explain in part the discrepancy in data surrounding miR-155, and underlines the importance of gaining further understanding of the roles that miRs play in different stages of cancer development.

The results in this chapter indicate that FOXP3 and the FOXP3-regulated miR, miR-7, both have growth suppressive activity in two aggressive breast cancer cell lines. Assays looking at the influence of miR-7 and miR-155 on the proliferative activity of the breast cancer cell lines show that miR-7 significantly reduces proliferation of both the MDA-MB-231 and BT549 breast cancer cell lines, while miR-155 has no significant effect on proliferation (Section 3.4.4). The lack of effect on proliferation seen upon miR-155 upregulation *in vitro* was interesting, as it has previously been proposed to have growth

promoting activity *in vitro* and *in vivo* (Mattiske et al., 2012, Wang and Hua, 2012). This is similar to previous reports, in which retroviral expression of miR-155 in the mouse 4T1 mammary cancer cell line did not result in any change in proliferative activity (Xiang et al., 2011). In this work, transfection with miR-7 resulted in reduced proliferative activity that was similar to that observed when the cell lines were transduced with *FOXP3*. Therefore it is possible that increased levels of miR-7 may drive the growth suppression observed in the *FOXP3*-transduced cell lines. Experiments to test this are described in Chapter 5.

Basement membrane extract (BME) invasion assays performed in the parental, control *GFP*-transduced and *FOXP3*-transduced breast cancer cell lines indicate that FOXP3 and FOXP3-regulated microRNAs can also suppress invasive migration (Section 3.4.5). The results of these experiments suggest that introduction of *FOXP3* into the BT549 and MDA-MB-231 cell lines results in a significant reduction in invasion, similar to the results seen when parental and *GFP*-transduced cell lines are transfected with miR-7. Interestingly, *FOXP3*-transduced cell lines that were also transfected with miR-7 showed the largest reduction in invasive ability. This raises the possibility that miR-7 and FOXP3 have independent functions, or that they work synergistically to further reduce invasion. MiR-7 has been implicated in the reduction of cell invasion, as it is known to regulate the pro-metastatic gene *PAK-1*. Given miR-155 is reported to suppress transcription factor 4 (TCF4) and epithelial-to-mesenchymal transition (EMT) in mouse breast cancer models (Xiang et al., 2011), it was interesting to observe that increased miR-155 had no effect on the basement membrane extract invasion assays. It is possible that further repeats of these experiments may have produced significant reductions in invasion upon miR-155 introduction. It is also possible that this particular experiment does not model the part of

metastasis in which miR-155 plays a significant role (such as epithelial-to-mesenchymal transition), and as such to assess this it is necessary to perform further investigations beyond the scope of this study.

Overall the results of this chapter support the hypothesis that FOXP3 and FOXP3-regulated miRs play a strong tumour suppressive role when expressed in breast cancer cell lines. Stable introduction of *FOXP3* results in the induction of two miRs that have established roles in breast epithelial cells, miR-7 and miR-155. Of importance, introduction of both *FOXP3* and miR-7 into breast cancer cells significantly reduces the cancerous phenotype of two highly aggressive breast cancer lines, suggesting that further investigation into the mechanisms involved in their tumour suppressive activity may identify novel targets for breast cancer subtyping and therapy.

CHAPTER 4: FOXP3-MEDIATED REPRESSION OF *SATB1*

4.1 Introduction

There is now an established role for FOXP3 as a tumour suppressor (Chen et al., 2008a, Ladoire et al., 2011, Martin et al., 2010, Liu et al., 2010), with the transcription factor able to act as both a transcriptional repressor and activator in epithelial cells. In the breast, FOXP3 has been shown to downregulate expression of the oncogenes *SKP2* and *HER2*, whilst maintaining expression of the *p21* and *LATS2* tumour suppressor genes (Liu et al., 2009a, Zuo et al., 2007a, Zuo et al., 2007b, Li et al., 2011b). This role as a tumour suppressor is not restricted to breast epithelial cells, with loss and mutation of *FOXP3* reported in prostate cancer, where FOXP3 has been found to directly repress transcription of *c-Myc*, an oncogene found to be highly overexpressed in a significant number of human cancers (Wang et al., 2009). More recently, FOXP3 has been linked to the p53 damage response, with p53 believed to induce *FOXP3* expression, with increased FOXP3 contributing to the growth suppressive activity of the p53 pathway (Jung et al., 2010).

In the previous chapter, two miRs, miR-7 and miR-155, were found to be upregulated by FOXP3 in the breast cancer cell lines MDA-MB-231 and BT549. Upregulated miR-7 was found to target components of the EGFR signalling pathway, including *EGFR*, *PAK-1* and *RAF-1*, and led to an inhibition of BT549 and MDA-MB-231 invasion and growth. Although miR-155 was also upregulated, the biological effect of this upregulation was not determined. MiRs are able to recognise loose seed sequences predominantly within the 3'UTR of target mRNA, and therefore have the potential to target multiple different transcripts (Krek et al., 2005) as demonstrated by large-scale identification approaches (Nikitina et al., 2012). This suggests that miR-7 and miR-155 target a number of transcripts in breast epithelia.

MiRs are believed to be involved in the regulation of well over half of the human transcriptome (Bartel, 2009, Rigoutsos, 2009). New evidence has revealed that miRs and transcription factors cooperate to create regulatory loops (Bartel, 2009, Brosh et al., 2008, Tsang et al., 2007, Croft et al., 2012), which include both feed-forward as well as feed-back loops. These regulatory loops enhance both the sensitivity and robustness of gene regulatory networks, as they involve regulation of a target via direct transcription factor binding, while also regulating the target via transcription factor-induced microRNAs (Filipowicz et al., 2008, Shalgi et al., 2007, Shimoni et al., 2007). This raised the possibility that FOXP3 and the miRs it regulates may form such regulatory loops within breast epithelia.

A common approach to identify potential targets is to use prediction programs that combine searches for a seed sequence complementarity, with evolutionary conservation among target sites between species, thermodynamic stability of the miR/mRNA hybrid and the presence of multiple target sites (Alexiou et al., 2009, Lhakhang and Chaudhry, 2012). However, not all miR targets predicted based upon complementarity with the miR seed sequence are functional targets (Thomson et al., 2011), suggesting that there are other factors that are involved which still need to be fully elucidated (Nikitina et al., 2012, Thomson et al., 2011). Therefore, any predictions must be verified experimentally.

The extent to which there is overlap between genes regulated by FOXP3 in Treg cells and breast epithelial cells was unknown at the start of this project; however the Treg studies indicated a large group of FOXP3 gene targets that have also been implicated in cellular growth and cancer (Sadlon et al., 2010, Pederson, 2007). The identification of FOXP3 targets by ChIP-seq in MCF-7 cells by Katoh *et al*. has supported this observation, with a large overlap (58.5%) in target genes observed between the cell types (Katoh et al., 2011).

Studies comparing Treg cell gene expression profiles with that of T helper cells have shown that *SATB1* expression inversely correlates with the expression of *FOXP3* (Beyer et al., 2011), suggesting that a similar phenomenon may be occurring in breast epithelial cells. Furthermore, *SATB1* was identified as containing multiple FOXP3 binding regions in genome-wide FOXP3 ChIP-on-chip experiments in Treg cells (Beyer et al., 2011, Sadlon et al., 2010), indicating that it was a potential direct target for FOXP3-mediated transcriptional regulation. Lastly, bioinformatics searches of target genes for miR-7 and miR-155 that show a negative correlation with FOXP3 in Treg cells identified *SATB1* as also being a potential target for both miR-7 and miR-155. This raised the possibility that multiple FOXP3-dependent mechanisms are employed by breast epithelia to ensure *SATB1* expression is normally repressed.

Recently, Special AT-rich Binding Protein 1 (*SATB1*) expression in breast cancer has been proposed to be an independent prognostic factor for breast cancer, with high *SATB1* mRNA and protein levels associated with advanced stages of breast cancer and poor prognosis (Han et al., 2008, Kohwi-Shigematsu et al., 2012, Patani et al., 2009, Li et al., 2010). As an oncogene, it is expressed in aggressive primary human breast epithelial tumours but it is not expressed in normal breast epithelia or non-metastatic breast cancers. Furthermore, high *SATB1* expression levels promote breast tumour growth and metastasis in *in vitro* and *in vivo* models of metastasis (Han et al., 2008). In clinical samples, *SATB1* protein expression has been correlated with poor patient prognosis. In addition, *SATB1* expression in human breast samples is increased in cases of chemotherapy-induced epithelial-to-mesenchymal transitions, metastasis and multi-drug resistance (Li et al., 2011a, Li et al., 2010). Together, these data indicate that induction of or increased expression of *SATB1* can be an important mechanism in the progression of cancer cells to a

more aggressive state. Therefore it is likely that *SATB1* levels within normal breast epithelia are under tight regulatory control (Patani et al., 2009).

This chapter demonstrates that *FOXP3* overexpression in the BT549 and MDA-MB-231 breast cancer cell lines can result in the suppression of the pro-metastatic oncogene *SATB1*. FOXP3 and FOXP3-regulated miRs, miR-7 and miR-155, are able to form a coherent feed-forward regulatory loop, by which they can bind to and suppress expression of *SATB1* at both a transcriptional and post-transcriptional level. This new mechanism for *SATB1* regulation may provide novel insights for the development of diagnostic tools and therapeutics for the treatment of breast cancers.

4.2 Aims and Hypothesis

The hypothesis for this chapter is that FOXP3 is able to regulate the expression of *SATB1* by binding to the promoter region of *SATB1* and also by regulating miRs that bind to the 3'UTR of *SATB1*.

The aims for this chapter were:

1. To confirm a direct interaction between FOXP3 and the 5' promoter region of *SATB1*

2. To confirm that miRs are able to bind to the 3'UTR of *SATB1* and consequently downregulate expression

3. To confirm that FOXP3 and FOXP3-regulated miRs can work together to robustly downregulate *SATB1* expression

4.3 Materials & Methods

4.3.1 Luciferase constructs

FOXP3-mediated repression of the human *SATB1* promoter was determined with promoter constructs kindly supplied by Professor Joachim Schultze and Dr Marc Beyer (University of Bonn, Bonn, Germany). Two FOXP3 binding regions (BR-1 and BR-2) identified by genome-wide FOXP3 ChIP (Figure 4.1) that were located in close proximity of the *SATB1* transcriptional start site were chosen for analysis. Briefly, the genomic DNA (626bp BR-1 and 379bp BR-2) surrounding the peak FOXP3 binding region were amplified by collaborators in Germany by PCR and cloned into the pGL4.24 vector (Promega, WI USA), upstream from a destabilised firefly luciferase and downstream from a minP element. To confirm the function of the FOXP3 binding sites within these regions, site-directed mutagenesis was performed to mutate consensus forkhead domain binding sites located in these regions (7 motifs in BR-1 and 6 motifs in BR-2). Consensus forkhead binding domains were identified using the transcription factor binding site identification tool MATinspector (Genomatix, http://www.genomatix.de). The *SATB1* promoter constructs were co-transfected with a pGL4.74 Renilla luciferase construct (Promega, WI USA) into parental, *GFP*-transduced and *FOXP3*-transduced BT549 or MDA-MB-231 cell lines to determine the response to FOXP3 using the Lipofectamine 2000 (Invitrogen, CA USA). Luciferase activity was determined 24 hours after transfection using a Promega dual luciferase kit, and analysis on a Veritas luminescent plate reader (Promega, WI USA) as per the manufacturer's instructions.

To determine the effect of miRs on the *SATB1* 3'UTR and to identify the functional miR binding sites within this region, the *SATB1* 3'UTR was amplified by PCR (Primers listed in Table 2.1) from the BT549 breast cancer cell line known to express high levels of *SATB1*

(Han et al., 2008) and cloned into a PsiCHECK-2 vector (Promega, WI USA) to make PsiCHECK-*SATB1*. Mutations in predicted miR binding sites identified by microRNA.org (http://www.microrna.org/microrna/home.do) were made by KAPA HiFi Hotstart mutagenesis PCR as per the manufacturer's protocol (KAPA Biosystems, MA USA). All PsiCHECK-*SATB1* reporter vectors were sequence verified, before HEK 293T or breast cancer cell lines were co-transfected with the reporter constructs and synthetic pre-miRs (10 μM) (Ambion, TX USA), using Lipofectamine 2000 transfection reagent (Invitrogen, CA USA). A non-targeting pre-miR (Ambion, TX USA) was used as a negative control. Transfections were performed in triplicate, with luciferase activity measured 24 hours later, as described above.

4.3.2 Transfection of breast epithelial cell lines with PNA miR inhibitors

Peptide Nucleic Acid (PNA) inhibitors (Panagene, South Korea) were used to block the activity of endogenous miRs. PNA inhibitors are DNA analogues in which the phosphate ribose ring of the DNA has been replaced with a polyamide backbone. These single stranded, chemically stable inhibitors are able to bind with sequence specificity to their target miR, thus preventing the miRs from associating with consensus seed sequences in mRNA targets (Oh et al., 2009).To measure the effect of miR knockdown in luciferase reporter assays, breast cancer cell lines were co-transfected with reporter plasmids and the PNA inhibitors (100 nM or 200 nM) using Lipofectamine 2000. All experiments were performed in triplicate in a 96 well format; with luciferase activity determined 24 hours later as described above. In experiments where the effect of PNA inhibitors on endogenous targets was tested, PNAs (final concentration of 100 nM or 200 nM) were transfected into cell lines using HiPerfect Transfection reagent. A non-targeting PNA inhibitor was used as a negative control, and transfection of BT549 and MDA-MB-231 cell lines was carried out

in 24-well plates or 6-well plates. Total RNA and whole cell lysate were collected from transfected cells 24 hours and 48 hours post transfection respectively. The knockdown of specific miR levels as a result of transfection with PNA inhibitors was determined by miR-specific Taqman RT-PCR.

4.4 Results

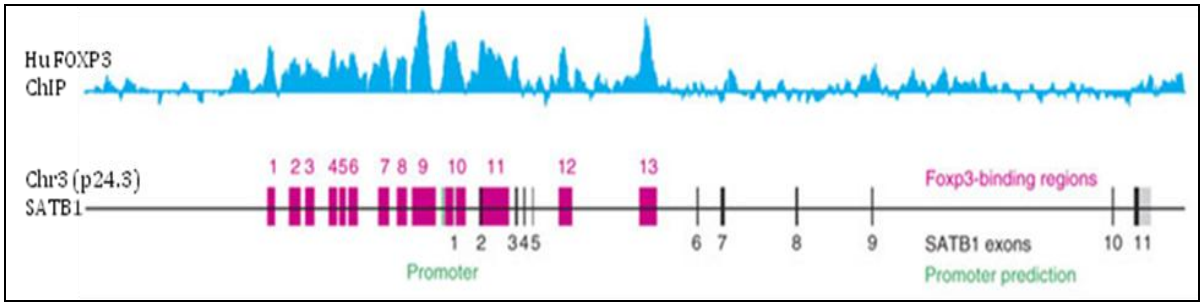
4.4.1 Identification of SATB1 as a potential target for a FOXP3-miR feed-forward regulatory loop

In Chapter 3, expression of FOXP3 in the BT549 and MDA-MB-231 breast cancer cell lines resulted in the upregulation of miR-7 and miR-155. Components of the EGFR signalling pathway, including *EGFR*, *RAF-1* and *PAK-1*, were shown to be downregulated by FOXP3, possibly through upregulation of miR-7, with miR-7 also shown to reduce growth and invasion of BT549 and MDA-MB-231. The biological outcome of miR-155 upregulation was not determined. As miRs recognise loose seed sequences in mRNA, with the potential to target multiple transcripts within the cell, it is likely that other gene transcripts are targeted by miR-7 and miR-155 within breast epithelial cells. The particular focus of this work was to identify feed-forward regulatory loops involving FOXP3 and FOXP3-regulated miRs.

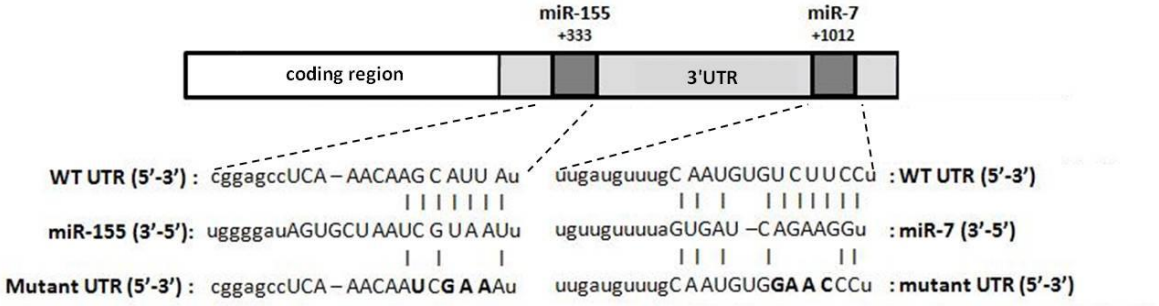
To investigate the mechanism of action of FOXP3 and its target miRs in breast epithelia, FOXP3 target gene lists that were generated in human Treg cells (Sadlon et al., 2010) were searched for genes that were also potential targets of FOXP3-regulated miRs. This search was restricted to those genes that showed both an inverse correlation with FOXP3 expression and known roles in cancer, apoptosis, cell growth and proliferation. Potential targets of miRs were identified using a selection of miR target prediction programs that are available on the miRGen website (http://www.diana.pcbi.upenn.edu/miRGen.html). A target was only considered to be of interest if it was identified as such by 3 separate miR prediction programs (miRanda, PicTar4 and TargetScan). From these investigations, one target that fit all of the criteria was the oncogene *SATB1*, which contains FOXP3 binding sites in the promoter region, while also containing predicted miR-consensus sequences for miR-7 and miR-155 in its 3'UTR (Figure 4.1).

Figure 4.1 ***Potential FOXP3 and FOXP3-regulated miR binding sites within the SATB1 gene and mRNA***

a)



b)



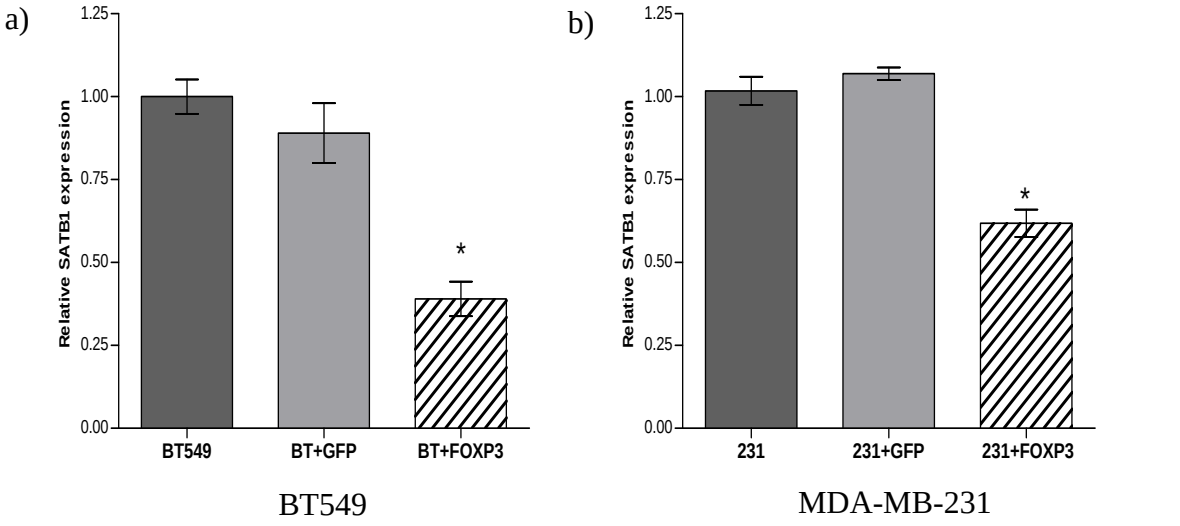
a) The gene structure of the human *SATB1* locus, showing the 11 exons within the gene (black) and the human FOXP3-binding regions (pink) that have been defined by ChIP analysis in human Treg cells, with 13 potential FOXP3-binding regions (1-13) annotated (Beyer et al., 2011). FOXP3 binding regions 9 and 10 were further investigated in these studies, and are henceforth referred to as BR-1 and BR-2 b) Annotation of the *SATB1* 3'UTR, showing predicted miR-155 and miR-7 seed sequences, and the experimental mutations introduced to these sequences.

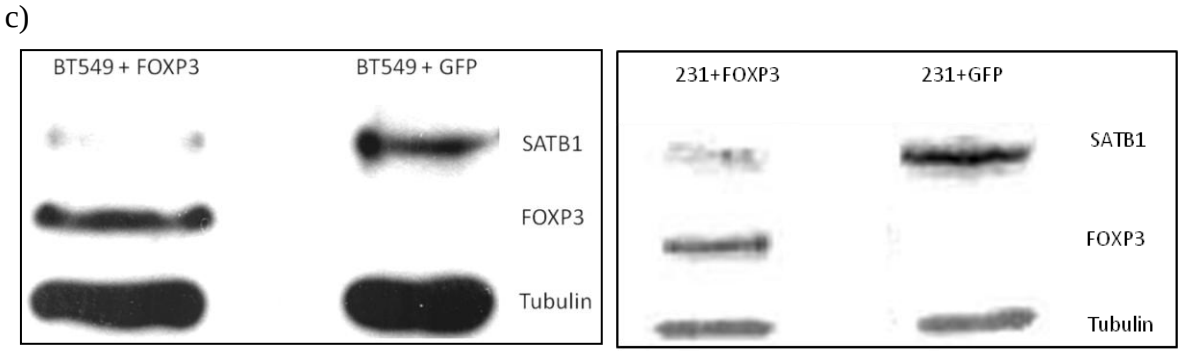
4.4.2 Overexpression of *FOXP3* results in reduced *SATB1* levels in breast cancer cell lines

To determine if *FOXP3* expression can influence *SATB1* levels, breast cancer cell lines were transduced with a lentivirus expressing the *FOXP3* gene (as described in section 3.4.1). The breast cancer cell lines used in these studies were BT549 and MDA-MB-231, as they express high levels of *SATB1* (Han et al., 2008), while expressing no or insignificant levels of *FOXP3* (Zuo et al., 2007b). Once stable expression of *FOXP3* had been established in the two cell lines, expression levels of *SATB1* as a result of *FOXP3* overexpression were determined by reverse transcription PCR (RT-PCR) and western blot analysis (Figure 4.2). Levels of *SATB1* mRNA were reduced by approximately 60% in *FOXP3*-BT549 cells when compared with either a *GFP*-transduced control line or the untransduced parental line ($P<0.0001$, $n=3$ independent transduction pools). Similar observations were made in the MDA-MB-231 cell line, with approximately 40% reduction in *SATB1* levels after transduction with *FOXP3* ($P<0.0001$, $n=3$ independent transduction pools) (Figure 4.2b)

The effect of *FOXP3* overexpression on endogenous SATB1 protein levels was determined by western blot analysis, which confirmed that the reduction in *SATB1* mRNA observed in the RT-PCR experiments resulted in lower protein levels. Overexpression of *FOXP3* in the BT549 cell line results in approximately 40% reduction in the levels of SATB1 protein, when compared with the control *GFP*-transduced lines (Figure 4.2c). Together these data indicate that *SATB1* levels are repressed in breast cancer cell lines when *FOXP3* is overexpressed.

Figure 4.2 ***Endogenous SATB1 is reduced when FOXP3 is overexpressed in BT549 and MDA-MB-231 cells***





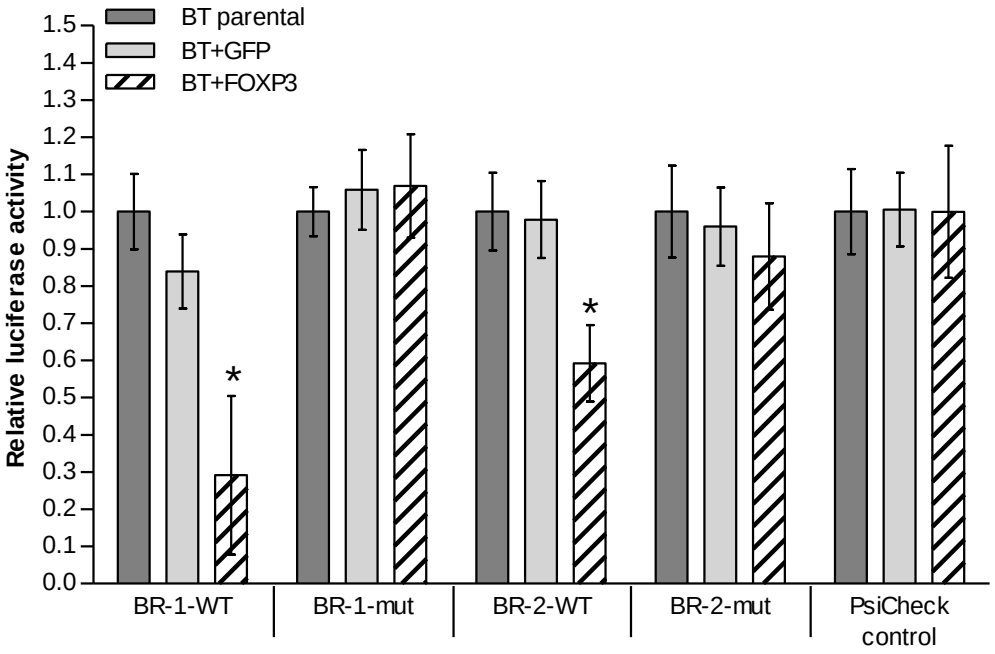
SATB1 mRNA levels in the parental (dark bars), *GFP*-overexpressing (grey bars) compared with *FOXP3*-overexpressing (hatched bars) BT549 (a) and MDA-MB-231 (b) cell lines as determined by qRT-PCR (n=3). cDNA (Quantitect cDNA kit, Qiagen, Germany) from total RNA (1 μg) was used in SYBR-Fast qRT-PCR *P=<0.05. c) SATB1 levels are reduced in *FOXP3*-expressing BT549 (left) and MDA-MB-231 (right) cells compared with control cells expressing *GFP*. Whole cell lysates (100 μg) were resolved on 4-15% PAGE gels, transferred to a nitrocellulose membrane and probed for SATB1 or FOXP3 with SATB1- or FOXP3-specific antibodies (listed in Table 2.1.2). Membrane was stripped with a Western Blot Recycling Kit (Alpha Diagnostics, TX USA) and re-probed with α –tubulin antibody as a loading control.

4.4.3 FOXP3 directly regulates transcription of *SATB1*

Previous ChIP-on-chip studies in human Treg cells identified a number of potential FOXP3 binding sites within the *SATB1* locus. In order to determine if FOXP3 is able to directly repress the *SATB1* promoter in breast cancer cells, *SATB1*-promoter luciferase constructs were tested for FOXP3-responsiveness in transient transfection assays. These constructs were kindly provided by Professor Joachim Schultze and Dr Marc Beyer (University of Bonn, Bonn Germany). Two predicted binding regions (BR-1 and BR-2) in *SATB1* located approximately -3kb and +0.5kb upstream and downstream of the *SATB1* transcription start site respectively were investigated separately. These constructs were transfected into parental BT549 breast cancer cells and control *GFP*- or *FOXP3*-transduced BT549 cells (as described in Section 3.4.1).

In BT549 cells transfected with the a *SATB1* promoter reporter construct containing either BR-1 or BR-2, a significant reduction in luciferase activity was only observed in *FOXP3*-transduced BT549 cells, but not in either *GFP*-transduced or untransduced control cell lines (Figure 4.3). Approximately 75% repression was observed for BR-1, while approximately 40% repression was observed for BR-2 (P=0.048). These findings indicated that both BR-1 and BR-2 contained functional FOXP3-responsive regions. Importantly, when the potential consensus FOXP3 binding sites within BR-1 or BR-2 binding regions were mutated, there was a loss of FOXP3-dependent reduction in luciferase activity, demonstrating that FOXP3-responsiveness required intact Forkhead DNA binding sites. No reduction in luciferase activity was observed when a control reporter construct lacking SATB1 regulatory sequences was transfected into any of the cell lines (control or *FOXP3*-transduced). These data together with the FOXP3 ChIP data indicate that FOXP3 is able to directly bind to and repress the *SATB1* promoter in breast cancer cells.

Figure 4.3 ***FOXP3 regulates the SATB1 promoter***



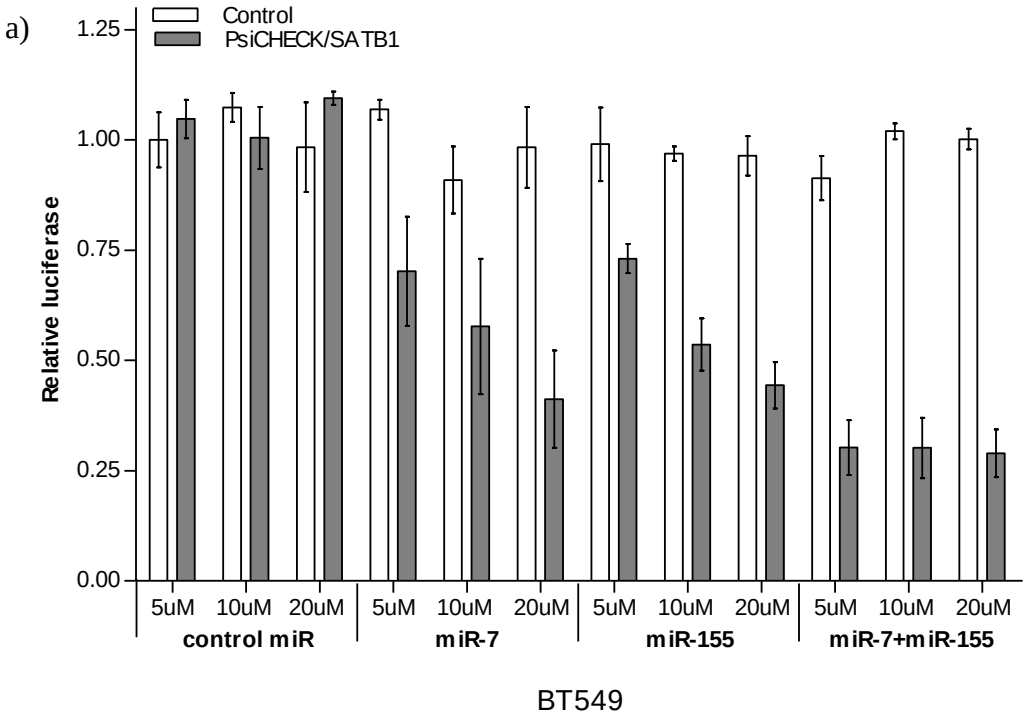
Luciferase reporter assays in BT549 cells using promoter constructs containing either wild-type FOXP3-binding sites at BR-1 and BR-2, or mutations of each region. Parental (dark bars), *GFP*- (grey bars) and *FOXP3*-transduced (hatched bars) cells were transiently co-transfected with a Firefly luciferase construct containing FOXP3 binding sites using Lipofectamine 2000 in triplicate wells. A vector that expresses a Renilla luciferase was used as a control for non-specific effects of *FOXP3* expression. Relative luciferase activity was normalised to Renilla activity. Mutant constructs consisted of BR-1 and BR-2 in which consensus FOXP3 binding site had been disrupted. Binding regions were mutated at the FOXP3 binding sites using mutagenesis PCR. When *FOXP3* is overexpressed in these cells the wild-type promoter is repressed, with repression lost by mutation of FOXP3 binding sites in either region. Data are expressed relative to the parental line and the *GFP*-overexpressing control cell line (n=3). *P<0.05.

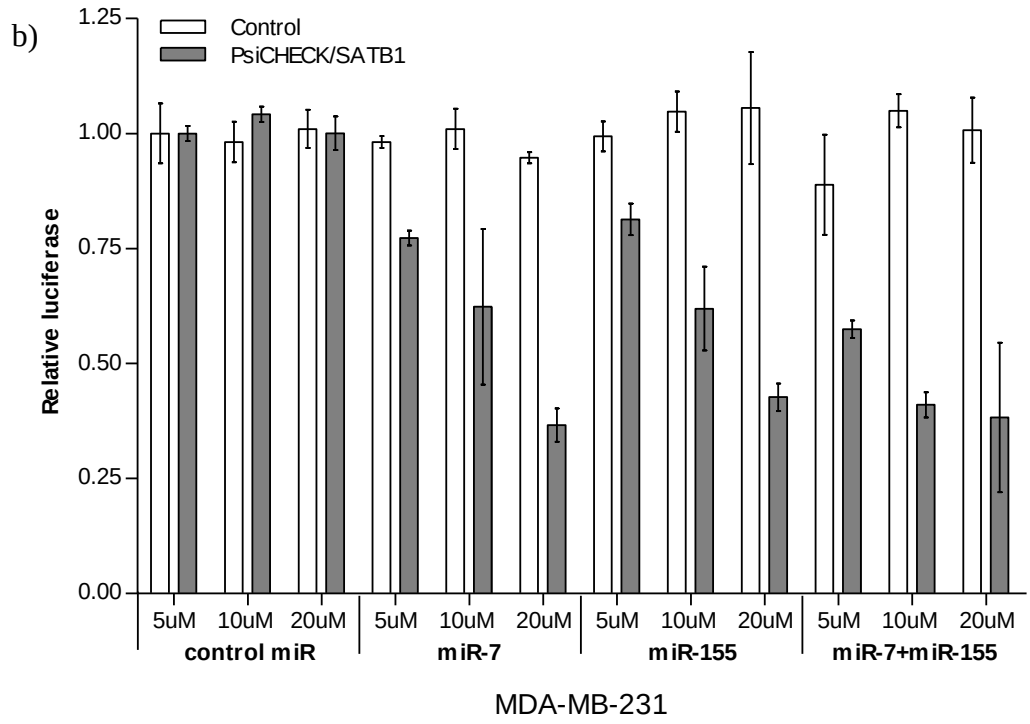
4.4.4 miR-7 and miR-155 downregulate expression of *SATB1*

The 3'UTR of *SATB1* was also identified by miR target prediction programs as containing potential single binding sites for the FOXP3-induced miRs, miR-7 and miR-155 (Figure 4.1b), raising the possibility that *SATB1* is also repressed by these miRs. To test the responsiveness of the *SATB1* 3'UTR to these miRs a *SATB1* 3'UTR luciferase reporter construct was made and luciferase activity determined in transient transfection assays (Figure 4.4).

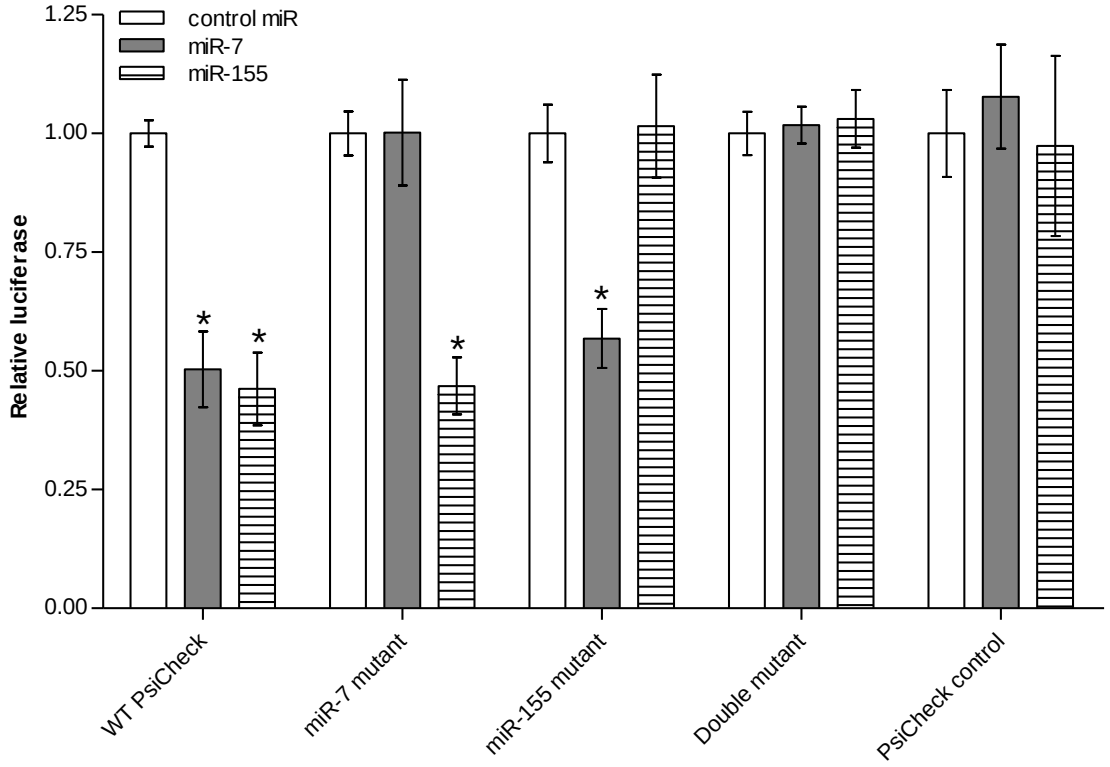
The *SATB1* 3'UTR reporter construct was co-transfected into the BT549 or MDA-MB-231 cell lines along with pre-miRs for either miR-7, miR-155, or a combination of both miRs, and a control non-targeting random sequence pre-miR (Figure 4.4a and b). A dose-dependent reduction in luciferase activity was observed when the BT549 cell line was transfected with miR-7 alone (30 to 65% reduction) or miR-155 alone (25% to 60% reduction). Interestingly, a 75% reduction in luciferase activity was observed in BT549 cells co-transfected with a combination of the two miRs (total concentration 5-15 μM range) irrespective of dose, suggesting that the two different targeting miRs can have a synergistic effect. No change in luciferase activity was observed in the cell line co-transfected with the control pre-miR, or when cells were transfected with a control PsiCHECK-2 luciferase construct lacking *SATB1* sequences. Similar observations were made in the MDA-MB-231 cell line, with dose dependent reduction in luciferase activity seen when co-transfected with miR-7 (25% to 70% reduction), miR-155 (20% to 60% reduction) or a combination of both miRs (45% to70% reduction). In this cell line the combination of miRs again caused a greater reduction in luciferase activity at lower doses than either pre-miR alone. For example, a 45% reduction in luciferase activity was

Figure 4.4 ***The SATB1 3'UTR is directly targeted by miR-7 and miR-155***





c)



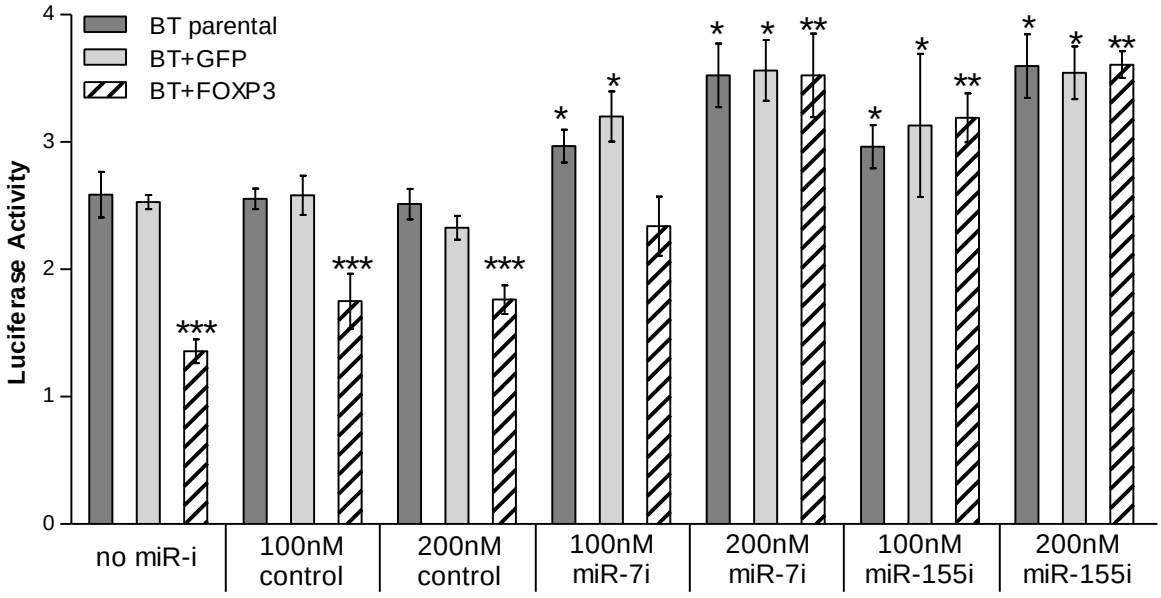
Transient expression of pre-miR-7 and pre-miR-155 in BT549 (a) and MDA-MB-231 (b) cells represses the luciferase activity from the *SATB1* 3'UTR construct, PsiCHECK-*SATB1*. Luciferase reporter constructs containing the *SATB1* 3'UTR (dark bars) or the reporter vector lacking the *SATB1* 3'UTR (white bars), were co-transfected into cells with increasing amounts of each individual miR (5 μM, 10 μM and 20 μM) or a combination of the miRs (equimolar mix of both pre-miRs to give the indicated total miR concentration). The results are normalized to the control pre-miR (n=3). c) Mutations of the miR-7 or miR-155 sites in the 3'UTR of the reporter vector were tested alone or in combination. BT549 cells were transfected with a control pre-miR (white bars), pre-miR-7 (dark bars) or pre-miR-155 (striped bars) (n=3, *P<0.05).

observed with the 5nM combination of both miRs (2.5 μM of each) compared with a 20% to 25% reduction observed with either miR alone. To confirm that these observations were due to direct binding of the miRs to the 3'UTR seed sequences, mutations were introduced into the miR consensus sequence sites located within the *SATB1* 3'UTR (Figure 4.1b) and the luciferase activity of these constructs was measured in response to increasing miR levels (Figure 4.4c). The mutant luciferase reporter constructs were co-transfected into the BT549 cell line along with the control pre-miR, pre-miR-7 or pre-miR-155. Mutation of either the predicted miR-7 or miR-155 consensus sites within the 3'UTR of *SATB1* blocked the ability of the corresponding pre-miR to downregulate luciferase activity, while mutation of both of the pre-miR sites prevented either miR from reducing luciferase activity. Together these transfection experiments strongly indicated that the 3'UTR of *SATB1* contains single functional binding sites for miR-7 and miR-155.

4.4.5 Endogenous miR-7 and miR-155 regulate levels of *SATB1* in the BT549 cell line

Since transiently transfected pre-miR-7 and pre-miR-155 were able to regulate the levels of the *SATB1* 3'UTR reporter construct, it was important to confirm that changes in endogenous levels of miR-7 and miR-155 were able to elicit a similar response in *SATB1* levels. To achieve this, Peptide Nucleic Acid (PNA) inhibitor molecules for miR-7 or miR-155 were used to block activity of their respective miRs. These PNA inhibitors, or a control, non-targeting PNA inhibitor, were co-transfected with the PsiCHECK luciferase reporter construct containing the 3'UTR of *SATB1*. Changes in luciferase activity in response to the PNA inhibitors were determined in the parental BT549 cell lines, *GFP*-transduced control BT549 cell lines and *FOXP3*-transduced BT549 cell lines (Figure 4.5). In the control *GFP* and parental cell lines, co-transfection of the 3'UTR construct with

Figure 4.5 ***miR targeting of SATB1 is blocked by PNA miR inhibitors***



Transient expression of PNA miR inhibitors (miR-i) targeting miR-7 or miR-155 in BT549 cells increases luciferase reporter activity from the PsiCHECK/*SATB1* 3'UTR reporter construct in a dose-dependent manner. Cells were co-transfected with reporter constructs and PNA miR inhibitors, with luciferase activity normalised to an internal firefly gene. Parental BT549 cells (dark bars) or BT549 cells expressing *GFP* (grey bars) show a miR-i-induced increase in luciferase activity when compared with the no miR-i control (*P<0.0001). This is also seen in cells overexpressing *FOXP3* (hatched bars) when compared with FOXP3 no miR-i control (**P<0.001). Luciferase activity was significantly reduced in *FOXP3*-expressing cells compared with parental or control *GFP*-transduced cell lines. ***P<0.05 compared with parental and *GFP* no miR-i control (n=3).

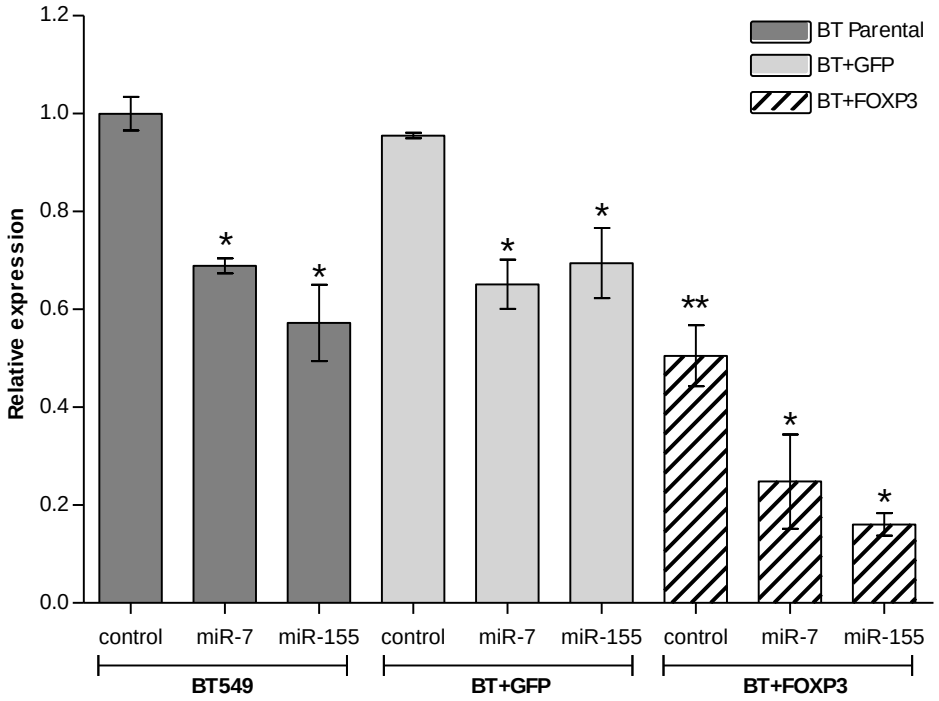
PNA inhibitors against miR-7 or miR-155 resulted in a 30-40% increase in luciferase activity, with no change observed when the control PNA was used. Of interest, in the BT549 cells overexpressing *FOXP3*, reporter activity was 45% lower than the control lines in the absence of the specific PNA inhibitors (***$p<0.05$), which is consistent with the previous observation of increased endogenous miR-7 and miR-155 expression in these cell lines (Section 3.4.3) as a result of *FOXP3* expression. Of importance, these *FOXP3*-expressing cell lines showed a significant dose-dependent, PNA-specific increase in the activity of the 3'UTR reporter constructs when compared with the no PNA inhibitor control (50 to 60% increase, **$P<0.001$). No significant changes in luciferase activity were observed when the control PNA inhibitors were used in *FOXP3*-expressing cells. This indicates that endogenous miR-7 and miR-155 have the potential to regulate levels of *SATB1* in breast epithelial cells.

4.4.6 MiR-7 and miR-155 are able to repress endogenous *SATB1*

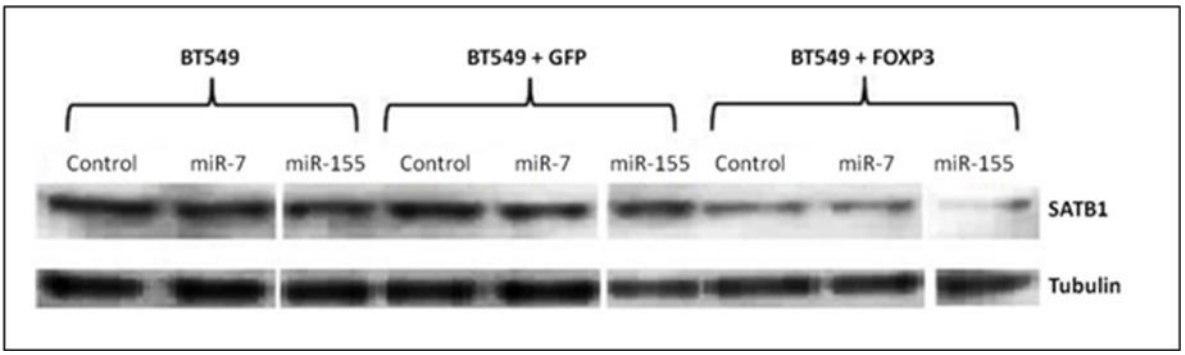
Although the experiments performed above confirmed that miR-7 and miR-155 were able to target the 3'UTR of *SATB1* when located in the 3'UTR region of the luciferase transcript, it was important to confirm that endogenous *SATB1* levels could be reduced by these miRs. This was determined by performing qRT-PCR and western blot analysis of *SATB1* expression in parental BT549, *GFP*-transduced control BT549 and the *FOXP3*-transduced BT549 cell lines transfected with control pre-miR, pre-miR-7 or pre-miR-155 (Figure 4.6a). Transfection of the control cell lines with either pre-miR resulted in a significant reduction in the *SATB1* mRNA levels when compared with the control pre-miR-transfected lines (35 to 38% reduction, $p=6.35\times10^{-5}$, and 35 to 45% reduction, $p=1.4\times10^{-9}$ respectively) as determined by qRT-PCR analysis.

Figure 4.6 ***Endogenous SATB1 expression is reduced by miR-7 and miR-155***

a)



b)



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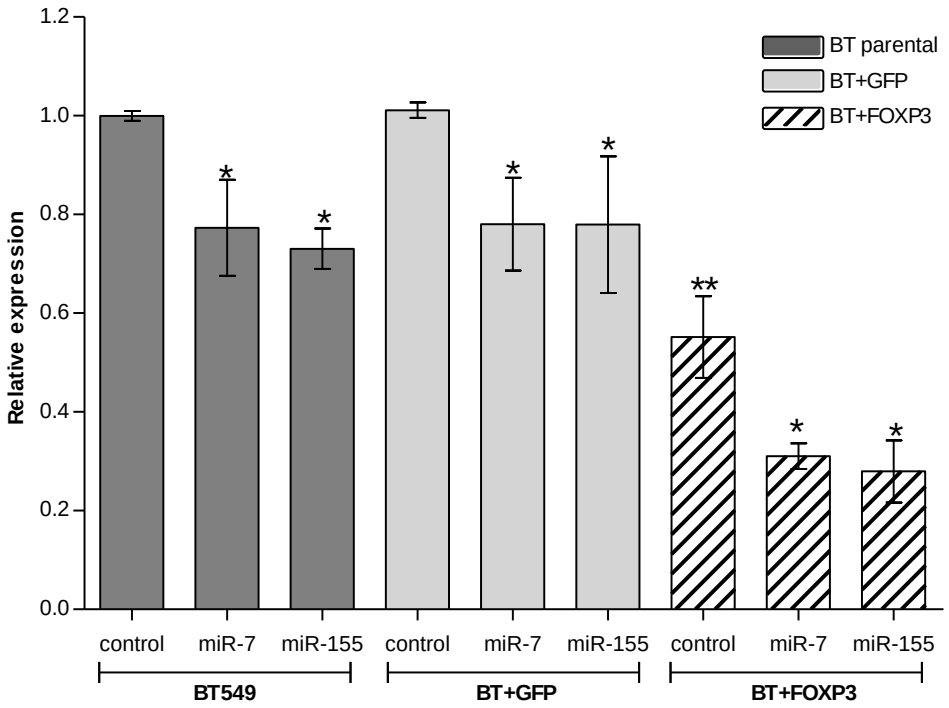


a) Expression of endogenous *SATB1* mRNA is reduced when pre-miR-7 or pre-miR-155 is transiently expressed in BT549 cells. *SATBl* levels in the parental cells (dark bars) and *GFP*-transduced control lines (grey bars) are reduced by the transfection of miR-7 or miR-155. Overexpression of *FOXP3* alone (hatched bars) reduces *SATB1* levels, compared with the control cell lines. Transient transfection of either pre-miR further reduces *SATB1* in these cells. (Triplicate RNA analysis of n=3 transfection pools, *$p<3.12x10^{-5}$, **$p=1.03x10^{-12}$) b) A representative SATB1 western blot of whole cell lysates from one of three independent transfection pools of BT549 cells. c) SATB1 protein band intensities were quantitated using ImageJ 1.43 (http://rsbweb.nih.gov/ij/) from images generated using the G:BOX iChemi imager (Syngene, Cambridge UK), and normalized to the tubulin loading control (n=3, *p<0.006, **p<0.01).

A significant reduction of endogenous *SATB1* levels was observed in the *FOXP3*-transduced BT549 cell lines transfected with the control pre-miR when compared with the control cell lines (45 to 50% reduction, $^{**}p=1.03x10^{-12}$). It was presumed that this was a result of both the direct repression of endogenous *SATB1* by FOXP3 (section 4.4.2 and 4.4.3), and miR-mediated repression of *SATB1* due to the FOXP3-dependent upregulation of the endogenous miR-7 and miR-155 (section 4.4.5 and 4.4.5). However, when the specific pre-miRs were transfected into the *FOXP3*-transduced BT549 cells, a further decrease in *SATB1* mRNA levels was observed (~30% reduction $^{*}p<3.12x10^{-5}$). When compared with the control *SATB1* levels in the parental and *GFP*-transduced BT549 cell lines, the additive effects of FOXP3 and either miR resulted in a 70 to 80% reduction in the message levels of *SATB1*.

These findings at the mRNA level were then confirmed at a protein level by performing western blots on whole cell lysates isolated from the cell lines transfected with control pre-miR, pre-miR-7 or pre-miR-155 (Figure 4.6b and c). In order to determine the relative quantities of protein from the western blots, three independent experiments were performed and protein densities analysed. The relative levels of SATB1 were normalised to the α-tubulin loading control protein. Consistent with the qRT-PCR determination of *SATB1* mRNA levels, a significant reduction in SATB1 protein levels was also observed as a result of miR-7 and miR-155 overexpression in all three of the cell lines tested. In addition, as observed in qRT-PCR, *FOXP3* expression alone resulted in reduced SATB1 protein levels which were further decreased when *FOXP3* and the miRs were overexpressed in the same cell line.

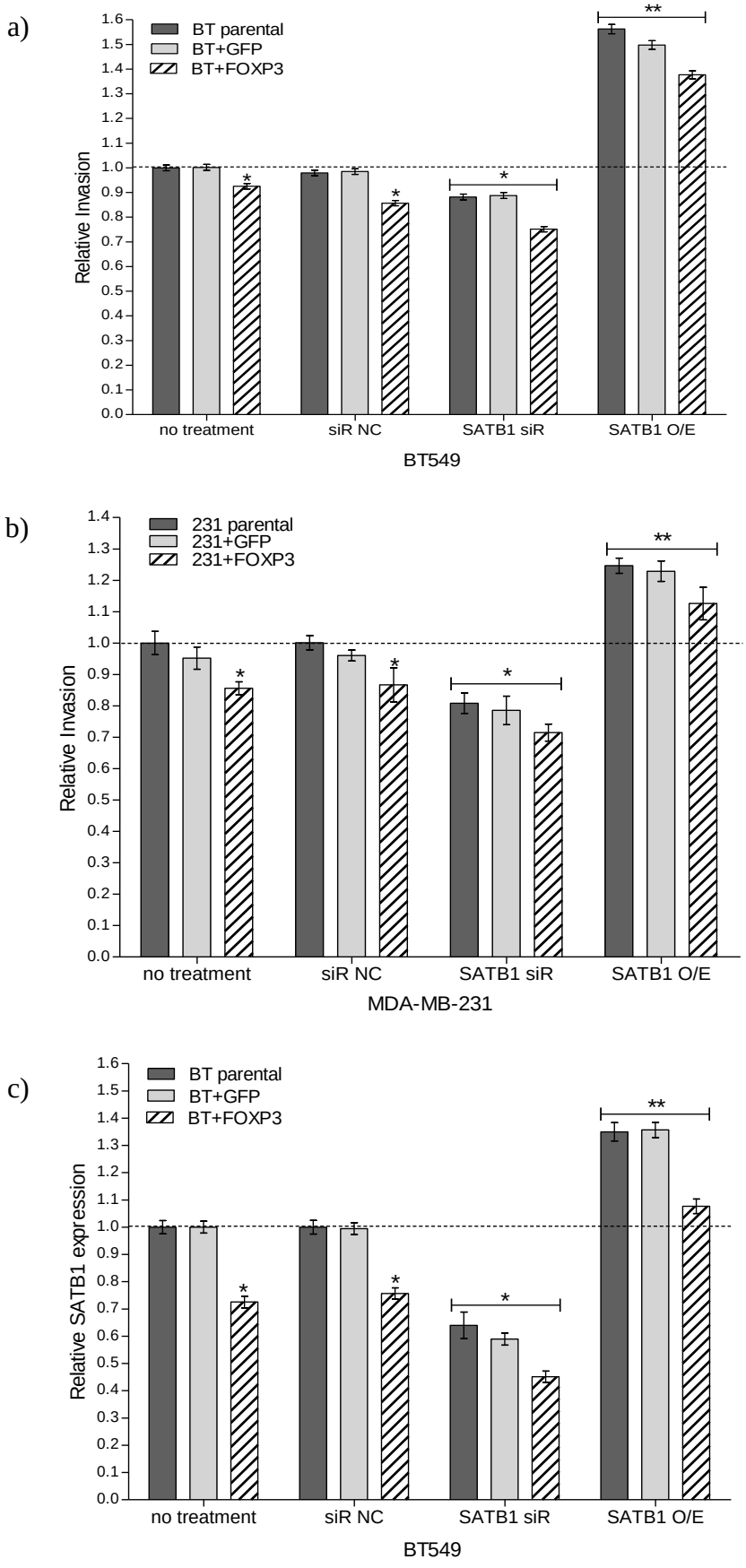
Taken together, these findings demonstrate that FOXP3 uses several mechanisms to suppress *SATB1* expression, including directly binding to the 5' *SATB1* regulatory regions and repressing transcription, while also acting indirectly by upregulating miRs-7 and -155 that are able to target the 3'UTR of *SATB1* message for post-transcriptional downregulation.

4.4.7 Altering *SATB1* levels affects the invasive potential of breast cancer cell lines

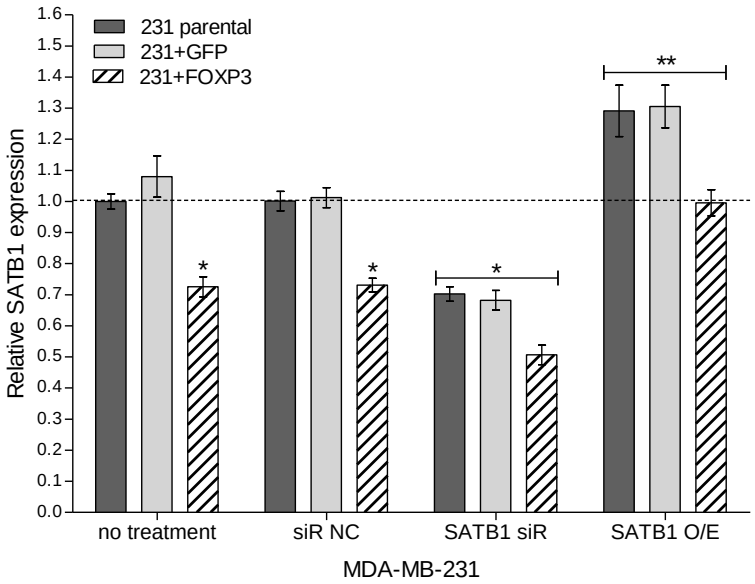
SATB1 is proposed to be a key oncogene involved in promoting the invasion and metastasis of breast cancers (Han et al., 2008, Li et al., 2010, Kohwi-Shigematsu et al., 2012, Patani et al., 2009) however this has recently been challenged (Hanker et al., 2011, Iorns et al., 2010). To determine whether *SATB1* levels can be correlated with invasiveness in the experimental system described in Section 3.3.4, the effect of manipulating *SATB1* levels or the levels of its upstream regulators FOXP3, miR-7 and miR-155 was determined in BT549 and MDA-MB-231 cells. Results in section 3.4.5 demonstrate that alteration of *FOXP3* and miR levels can result in a reduction in invasive potential of the BT549 and MDA-MB-231 cell lines. To determine if this reduction was in part due to the alteration of *SATB1* levels, either a *SATB1* overexpression construct or *SATB1* siRNA were transiently transfected into the BT549 and MDA-MB-231 breast cancer cell lines, and these cells were then tested in the BME cell invasion assay (Figure 4.7).

No significant differences in invasive activity were observed between the no treatment cell lines and the siRNA negative control cell lines. In parental and control *GFP*-transduced cell lines transfection with a *SATB1* siRNA caused a significant 10% or 20% reduction in

Figure 4.7 ***Invasive ability is affected by altering levels of SATB1***



d)



Basement membrane extract (BME) invasion assays of parental (dark bars), *GFP*-transduced (grey bars) and *FOXP3*-transduced (hatched bars) BT549 (a) and MDA-MB-231 (b) cells. A significant reduction in invasive potential (~10%) is observed when *SATB1* expression is reduced with an siRNA (*SATB1* siR) when compared with the control no-treatment and negative control siRNA (siR NC) cells. *SATB1* overexpression via transient transfection with an expression construct (*SATB1* O/E) significantly increased invasion, with an approximate 50% and 25% increase observed in parental and control GFP-transduced BT549 and MDA-MB-231 cell lines respectively. N=4, *p<0.02, ** p<0.0002. Relative *SATB1* expression levels of parental (dark bars), *GFP*-transduced (grey bars) and *FOXP3*-transduced (hatched bars) BT549 (c) and MDA-MB-231 (d) cells, as determined by RT-PCR. *SATB1* levels are shown relative to the parental, no treatment cell line. N=3, *p<0.01, **p<0.0001

invasive ability in BT549 and MDA-MB-231 cell lines respectively ($p<0.02$). Conversely, cells that were transiently transfected with a *SATB1* expression vector showed a significant increase in invasion when compared with the control cell lines. In the BT549 cell lines, a 50% increase in invasive ability was observed upon introduction of *SATB1*, while in the MDA-MB-231 cell line, introduction of *SATB1* resulted in a 25% increase in the amount of invasion ($p<0.0002$). As observed in section 3.4.5, transduction with *FOXP3* results in significantly reduced levels of invasion when compared with *GFP*-transduced control and parental cell lines (10 to 15% reduction, $p<0.02$). Importantly, *SATB1* knockdown with siRNA caused a further decrease (10%) in the invasive potential of both *FOXP3*-transduced BT549 and MDA-MB-231 cells. In contrast, overexpression of *SATB1* increased the invasive potential of the cell lines (50% increase in BT549 cells and 25% increase in MDA-MB-231 cells), indicating that forced expression of *SATB1* can overcome the inhibitor effect of FOXP3. These results suggest that both FOXP3 and SATB1 have the ability to influence the invasive potential of breast cancer cell lines, with FOXP3 acting to reduce invasive potential, while SATB1 acts to increase it. This suggests a potential role for FOXP3 in controlling *SATB1* levels so as to prevent inappropriate migration and epithelial-to-mesenchymal transition.

4.5 Discussion

Recently it has been proposed that FOXP3 is able to function as a tumour suppressor in breast and prostate epithelium (Martin et al., 2010, Wang et al., 2009, Zuo et al., 2007a, Zuo et al., 2007b), although the molecular pathways and targets in these cell types are still largely unknown. This chapter demonstrates that *SATB1* is repressed both directly and indirectly by FOXP3 in breast cancer cell lines. FOXP3 was found to be able to bind directly to the *SATB1* promoter region (sections 4.4.2 and 4.4.3). This thesis has focussed on 2 binding regions within close proximity of the *SATB1* promoter, however other functional FOXP3 binding regions within the *SATB1* gene have been established in Treg cells (Beyer et al., 2011). Therefore it would be of interest to determine if these binding regions are also functional within breast epithelia. Previous studies in Treg cells have established that FOXP3 can function to regulate targets through interactions with histone modifiers (Li et al., 2007, Beyer et al., 2011), or through interference of transcription factor function (Bettelli et al., 2005), and therefore it is possible that a similar process is occurring to suppress *SATB1* in breast epithelia.

Not only has this work shown that FOXP3 is able to regulate *SATB1* by directly binding to the promoter region, but has also demonstrated that FOXP3 upregulates 2 microRNAs, miR-7 and miR-155, which can target the *SATB1* 3'UTR for further regulation (Sections 4.4.4-4.4.6). Both of these miRs were able to significantly suppress levels of *SATB1* independently of FOXP3. Interestingly, when a low dose combination of miRs was co-transfected into the BT549 and MDA-MB-231 breast cancer cell lines with a *SATB1* 3'UTR luciferase reporter, a larger reduction in luciferase activity was observed than seen when transfected with higher doses of the individual miRs. This suggests that these miRs have an additive functional effect on *SATB1*, however as these experiments were

performed using hybrid constructs this requires further investigation in an endogenous setting. It is expected that miRs will have an additive effect, as target mRNAs usually have target sites for more than one miR, and in many cases can have multiple target sites for a single miR, allowing for more robust regulation (Bartel, 2009).

Importantly, stronger suppression of endogenous *SATB1* was observed when FOXP3-transduced cells were transfected with pre-miR-7 or pre-miR-155 compared with cells only transduced with *FOXP3*, suggesting that the levels of miRs delivered by transient transfection may be well above normal physiological levels. A low level of SATB1 may therefore be required within the cell. Consistent with this, low levels of SATB1 were observed in later experiments performed in HMECs (chapter 5). FOXP3, miR-7 and miR-155 expression was also shown to have a greater effect on reducing *SATB1* mRNA levels than protein levels, however this is most likely due to experimental timing, as the reduced transcript levels may not result in fully reduced protein levels within the 48 hour time frame examined. Furthermore, *in vitro* BME invasion assays carried out in cell lines in which *SATB1* levels have been manipulated (Section 4.4.7) support the recent proposal that *SATB1* is an oncogene involved in invasion and metastasis, with overexpression of *SATB1* leading to increased invasion of breast cancer cell lines. Together, the evidence in this chapter suggests that part of FOXP3 tumour suppressor function involves repressing the metastasis-promoting oncogene *SATB1*. Further work involving *in vivo* and *in vitro* assays testing cell lines with manipulated *SATB1* levels are required to fully support this hypothesis.

Although overexpression of *SATB1* in epithelial cells has been associated with cancer progression (Han et al., 2008, Kohwi-Shigematsu et al., 2012, Lu et al., 2010b, Patani et al., 2009), it is also known to play an important role in regulating gene expression in T cell development (Alvarez et al., 2000, Yasui et al., 2002). Studies into the structure and functional analysis of the mature SATB1 protein predict that it contains a PDZ-like signalling domain, a CUT repeat domain and a homeodomain. The PDZ-like signalling domain is believed to be critical for SATB1 activity, as it is located within the N-terminal region and provides a dimerization interface, while also allowing SATB1 to interact with multiple proteins, including co-repressors and co–activators (Cai et al., 2006, Yasui et al., 2002). SATB1 appears to be able to regulate transcription, both by acting as a global modifier of chromatin architecture (Cai et al., 2003) and by acting as a transcription factor, recruiting co-repressors or co-activators directly to promoters (Pavan Kumar et al., 2006). SATB1 binds to AT-rich sequences of DNA in the genome, and can then tether these DNA sites to cage-like nuclear networks that surround heterochromatin. These anchorage sites then recruit chromatin remodelling enzymes, allowing SATB1 to create a higher-order chromatin structure, and by dynamically arranging genes in loop domains, regulating histone modifications and nucleosome positioning (Alvarez et al., 2000, Cai et al., 2006). The organisation of these loop domains is specific to the cell type (Cai et al., 2003). SATB1 function is best characterised in T cells, where its role in T cell development was uncovered by gene knockout studies (Alvarez et al., 2000). Mice lacking *Satb1* had significantly smaller thymus, spleen and lymph nodes when compared with wildtype mice, and died on average at 3 weeks of age. This was due to a significant reduction in immature T cells, and T cell apoptosis as a result of increased expression of genes (including proto-oncogenes, cytokine receptor genes and apoptosis genes) at inappropriate stages of T cell development (Alvarez et al., 2000). This suggested that lack of *Satb1* expression results in irreversible perturbation of thymocyte development and function (Alvarez et al., 2000).

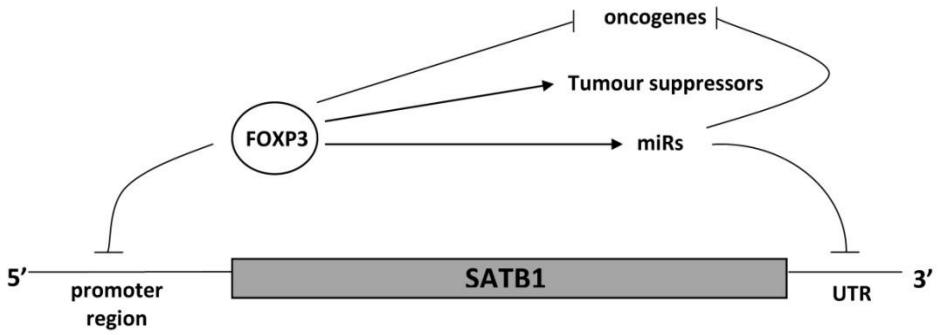
Satb1 has also been implicated in regulating multiple signalling pathways, including the Notch, Wnt and TGFβ pathways, extracellular attachment, cell integrity and cell structure (Naito et al., 2011, Xue and Zhao, 2012, Li and Flavell, 2008, Fehérvari and Sakaguchi, 2004). On a broader gene scale, it was found that Satb1 is responsible for the regulation of more than 10% of genes expressed in T cells and cancerous breast cancer cells lines, supporting a role as a global regulator (Han et al., 2008).

SATB1 was first linked to breast cancer metastasis due to its high expression levels in aggressive breast cancer cell lines, but low to no expression in normal and immortalised human mammary epithelial cells (Han et al., 2008). Analysis of human breast cancer samples by immunohistochemistry associated nuclear SATB1 expression with poorly differentiated cancers, while normal adjacent tissue lacked SATB1 expression (Han et al., 2008). Subsequent investigation demonstrated that breast cancer cells with high levels of the *SATB1* oncogene can undergo extensive changes to their gene expression profile, resulting in the cells acquiring a metastatic phenotype (Han et al., 2008). The work in this thesis, in addition to work by others, supports a pro-cancerous role for SATB1 in breast cancer, a role which is also observed in other cancers, including in melanoma, gastric cancer and rectal cancer (Cheng et al., 2010, Kohwi-Shigematsu et al., 2012, Lu et al., 2010b, Patani et al., 2009, Chen et al., 2011, Meng et al., 2012). In addition to promoting metastasis, SATB1 has also been associated with the acquisition of multidrug resistance in human breast cancers, which it achieves partially through the prevention of cell apoptosis (Li et al., 2010). Although other studies using RNA expression analysis have argued against the prognostic value of SATB1 in human breast cancer samples (Hanker et al., 2011, Iorns et al., 2010), it is possible that contamination of the cancer samples with tumour infiltrating cells such as stromal cells and T lymphocytes may have influenced

SATB1 mRNA quantitation (Iorns et al., 2010, Kohwi-Shigematsu et al., 2010). Critically, an association between *SATB1* expression and prognosis cannot be assessed purely based on the expression of *SATB1* at the transcript level, as *SATB1* expression is not limited to cancer cells (Kohwi-Shigematsu et al., 2010). It is also important to note that *SATB1* mRNA levels do not necessarily equate directly with the levels of SATB1 protein, as SATB1 levels can also be regulated post-transcriptionally by miRs, as observed in this work and others (Li et al., 2011a). Iorns *et al* also reported that levels of *SATB1* mRNA do not change in human breast cancer cell lines compared with non-tumourigenic cell lines, and thus is not associated with breast cancer progression (Iorns et al., 2010), however large differences in *SATB1* expression levels have since been reproducibly detected at both the message and protein level in this work and other studies comparing breast cancer cell lines with healthy breast epithelial lines (Kohwi-Shigematsu et al., 2012, Patani et al., 2009). One possible reason for this discrepancy raised by Kohwi-Shigematsu is that this difference is due to the probe sequences used in the hybridisation microarray studies of Iorns *et al.* not being *SATB1*-specific, as there is significant overlap with the sequence of *SATB2* (Kohwi-Shigematsu et al., 2010).

Although there has recently been increased interest in the role of the *SATB1* oncogene, there is still little known about how the gene is regulated, or how it is induced in the transition from normal to cancerous breast epithelium. In particular, the mechanism by which *SATB1* expression is upregulated in cancer is unknown. This chapter contains novel data demonstrating that in highly aggressive, late stage breast cancer cells that overexpress *SATB1*, FOXP3 and FOXP3-regulated miRs can function in a feed-forward regulatory loop model to suppress this expression (Figure 4.8). It is as yet unknown whether a principal function of FOXP3 and FOXP3-regulated microRNAs in normal epithelial cells is to

Figure 4.8 ***A model of feed-forward regulation of SATB1 by FOXP3***



A schematic representation of the feed-forward regulation of *SATB1* by FOXP3. FOXP3 acts directly at the 5' promoter region, but also functions indirectly by inducing miRs that target the 3'UTR of *SATB1*. FOXP3 may induce other tumour suppressors and repress other oncogenes to give tight regulation of the target protein.

prevent the expression of *SATB1*. If this is the case, it does not explain why in less aggressive breast cancer cells that have lost FOXP3 there are still low levels of *SATB1*. One explanation is that there are other mechanisms involved in the upregulation and suppression of *SATB1*. Recently, miR-448 has been identified as being involved in the repression of *SATB1* (Li et al., 2011a), and as shown in this thesis with miR-7 and miR-155, loss of miR-448 also leads to the upregulation of *SATB1* (Li et al., 2011a). Together this evidence suggests that there are multiple changes required before overexpression of *SATB1* in normal breast tissue can occur, and this therefore requires further investigation.

CHAPTER 5: FOXP3 EXPRESSION IN NORMAL BREAST EPITHELIA

5.1 Introduction

To this point, the work outlined in this thesis has examined the effect of expression of a *FOXP3* transgene in breast cancer cell lines. This chapter investigates the role of FOXP3 in normal breast epithelia and how loss of *FOXP3* contributes to the progression of breast cancer. Although a great deal is known about the factors that regulate *FOXP3* expression in T regulatory cells, little is known about its regulation in breast epithelia. Many of the transcription factors and pathways that are involved in *FOXP3* regulation in Treg cells are also thought to be active in breast epithelia, such as the c-Rel (Fullard et al., 2012), SMAD (Sundqvist et al., 2012), and Runx (Ito, 2008) transcription factors, suggesting that these factors may also have a role in FOXP3 transcriptional regulation in epithelia. Recently, Liu *et al* have proposed a role for an Activating Transcription Factor (ATF-2)/c-Jun heterodimer in *FOXP3* transcription in breast epithelia (Liu et al., 2009b). These transcription factors were shown to induce the transcription of *FOXP3* in breast cancer cell lines in response to the drug Anisomycin, through a c-Jun-NH_2-kinase dependent pathway. Anisomycin treatment of two breast cancer cell lines, BT474 and MCF-7, resulted in a significant reduction in cell viability, due to the induction of p53-independent apoptosis (Liu et al., 2009b). This induction of apoptosis was brought about by an increase in *FOXP3* expression. Anisomycin was also shown to confer a significant therapeutic effect on established mammary tumours in mice, resulting in a reduction of tumour growth that is dependent on *FOXP3* expression (Liu et al., 2009b).

Recently, *FOXP3* has been proposed to be directly regulated by the transcription factor p53, forming a component of the p53 growth arrest response to DNA damage (Jung et al., 2010). The *p53* transcription factor is activated in response to genotoxic and oncogenic stress, resulting in the upregulation of genes that are involved in cellular responses such as

apoptosis and cell cycle arrest (Vousden and Lu, 2002, Vousden, 2002, Harris and Levine, 2005). *p53* functions as a tumour suppressor, with mutations seen in many human cancers. These include inactivating mutations, but also gain of function mutations, resulting in the inability of cells to maintain normal damage responses.

A link was first made between *FOXP3* and *p53* when known *p53* regulatory targets *HER2* and *SKP2* (Tang et al., 2004, Hu and Aplin, 2008) were shown to also be transcriptional targets of FOXP3 (Zuo et al., 2007a, Zuo et al., 2007b). Using DNA damage agents, Jung *et al.* demonstrated that stabilisation of *p53* via the DNA damage response also resulted in *FOXP3* induction in breast and colon cancer cell lines (MCF-7 and HCT116). Of importance, when a *p53*-specific siRNA was transfected into cells treated with the DNA damaging agent Etoposide, induction of *FOXP3* in response to treatment was almost entirely abrogated (Jung et al., 2010). Treatment with Nutlin-3a, which results in the stabilisation and accumulation of p53 via inhibition of *Mdm2* (Miliani de Marval and Zhang, 2011), in the absence of DNA damage also led to the induction of *FOXP3* in MCF7 and HCT116, indicating that *p53* accumulation and hence transcriptional activity is sufficient for *FOXP3* expression. Further investigation of protein expression via immunostaining of human breast and colon cancer cell lines revealed co-localisation of *FOXP3* with p53 in the nuclei of cells that were exposed to a DNA damaging reagent, suggesting that p53 is a key regulator of *FOXP3* expression during DNA damage responses (Jung et al., 2010). Lastly, *FOXP3* induction was proposed to be an important downstream mediator of the p53 growth arrest pathway, as abolishing FOXP3 induction in etoposide-treated cells with a *FOXP3* siRNA significantly reduced the p53-mediated damage response (Jung et al., 2010).

These findings linking FOXP3 with DNA damage pathways have potential clinical significance, as shown by a recent retrospective study in the PACS01 clinical trial (Ladoire et al., 2012). In this retrospective study of 692 $FOXP3^-$ and 405 $FOXP3^+$ breast cancer samples, adjuvant chemotherapy treatment was found to result in greater efficacy of treatment and overall survival in patients with nuclear *FOXP3* expression (P=0.003) (Ladoire et al., 2012). The linkage of FOXP3 to DNA damage responses, growth suppression and increased apoptosis has led to efforts to increase *FOXP3* expression in tumours. Sequencing of breast cancer samples has shown that unlike the expressed allele of *FOXP3* which is mutated, the copy of *FOXP3* on the inactive X chromosome is normally wildtype (Zuo et al., 2007b). This presumably reflects the fact that it has not been under any selection pressure. In other cases, *FOXP3* expression appears to be significantly downregulated. As *FOXP3* is not irreversibly inactivated in a large proportion of breast cancers (Zuo et al., 2007b), it is possible that reactivation of wildtype *FOXP3* may be an effective therapeutic for the treatment of particular breast cancer subtypes (Jung et al., 2010, Liu et al., 2009b). As mentioned, Anisomycin has been used to activate *FOXP3* in *in vitro* and *in vivo* mouse models, while HDAC inhibitors have been found to induce *FOXP3* in human breast cancer cell lines *in vitro*, leading to increased apoptosis (Ladoire et al., 2012). Treatment of syngeneic BALb/c mouse mammary cancer models with Anisomycin has been shown to result in a significant reduction in tumour size when compared with mice treated with a vehicle control (100 mm^3 vs. 1200 mm^3 respectively), although no comment was made as to whether this was a direct result of *FOXP3* induction (Liu et al., 2009b). Although these results are promising, only 5 mice were analysed for each treatment group, and therefore further investigation is required to evaluate the potential of Anisomycin-dependent activation of *FOXP3* as a therapeutic for breast cancer.

This chapter demonstrates that *FOXP3* is expressed in normal mammary epithelial cells, where it maintains miR-7 and miR-155 expression. Using the benign, immortalised mammary epithelial cell line MCF10a, the role of FOXP3 and FOXP3-induced miR-7 in p53- and Anisomycin-induced apoptosis was investigated. The results from this work suggest that *FOXP3* and miR-7 induction or activation may be suitable approaches for the treatment of breast cancers.

5.2 Aims and Hypothesis

The hypothesis for this chapter is that *FOXP3* expression, through the regulation of miR-7, plays a role in restricting growth and response to mitogens in normal epithelia. Increased expression of FOXP3 and miR-7 levels, potentially via the DNA damage pathway, can alter the balance between survival and apoptosis within cells.

The aims of this chapter are:

1. To confirm that *FOXP3* is expressed in normal breast epithelial cells and that FOXP3 regulates the expression levels of miR-7 and miR-155 in these cells.

2. To confirm the induction of *FOXP3* with genotoxic reagents Nutlin-3a and Etoposide

3. To confirm a role for FOXP3 and FOXP3-regulated miRs in the induction of apoptosis

5.3 Materials & Methods

5.3.1 HMEC and MCF10a cell lines

The adherent human mammary epithelial cell (HMEC) line is derived from primary normal breast epithelial cells that have been isolated from glandular tissues of the breast (Lonza, Switzerland). These cells are the closest defined system available to model normal human breast epithelial cells *in vitro* (Hinshelwood and Clark, 2008). The MCF10a cell line is an adherent, non-tumourigenic breast epithelial cell line (ATCC, VA USA) that was produced through the long-term culture of human epithelial cells in serum-free medium containing a low concentration of Ca^{++}. These cells are non-tumourigenic and immortalised, showing no signs of terminal differentiation or senescence, and are a suitable model for studying human breast cancer progression (Zientek-Targosz et al., 2008).

5.3.2 Nutlin-3a and Etoposide treatment of normal and immortalised breast epithelial cell lines

Genotoxic agents Nutlin-3a and Etoposide (Sigma-Aldrich, MO USA) were used for the induction of *p53* expression in the MCF10a immortalised breast epithelial cell line. Nutlin-3a is able to induce the stabilisation and accumulation of p53 by inhibiting the Murine double minute (*Mdm2*) oncogene (Thompson et al., 2004). Etoposide is a chemotherapeutic that functions by forming a complex with DNA and topoisomerase II. This leads to damage of the DNA strands, subsequent disruption of DNA synthesis and the promotion of apoptosis via the p53 pathway (Chresta et al., 1996).

In the Nutlin-3a experiments, two concentrations of Nutlin-3a were tested which had been shown to induce *FOXP3* expression previously in the MCF-7 and HTC118 cell lines (Jung et al., 2010). Cells were seeded at $5x10^5$cells/flask in T25 flasks or at $3x10^5$cells/well in 6

well plates the day prior to Nutlin-3a treatment for protein and RNA isolation respectively. For Nutlin-3a treatment, medium was removed and replaced with fresh, untreated medium or medium containing Nutlin-3a at a concentration of 5 μM or 10 μM. Cells were incubated at 37^{o}C/5%CO_2 and RNA and whole cell lysate was extracted from the cells 24 hours and 48 hours after treatment, and used for qRT-PCR and western blots respectively.

In Etoposide treatment experiments, cells were seeded the day before treatment as for the Nutlin-3a experiments above. The following day, medium was removed and replaced with fresh, untreated medium or medium containing Etoposide at a concentration of 20 μM. Cells were incubated at 37^{o}C/CO_2 and RNA and whole cell lysate was isolated from cells 24 hours and 48 hours post-treatment and was used for qRT-PCR and western blots experiments.

5.3.3 Anisomycin treatment of normal and immortalised breast epithelial cell lines

Anisomycin (Sigma-Aldrich, MO USA) is an antibiotic drug commonly used to activate MAP kinases, stress-activated protein kinases (including the c-Jun N-terminal kinase/stress activated protein kinase signalling pathway), and a number of other signal transduction pathways (Cano et al., 1994). It has recently been shown to activate *FOXP3* expression in normal and cancerous breast epithelial cells via a JNK and c-Jun/ATF2 dependent pathway (Liu et al., 2009b).

The ability of Anisomycin to re-activate *FOXP3* expression was tested in the immortalised (MCF10a) breast cell lines. MCF10a cells were seeded the day before treatment at

$5x10^5$cells/flask in T25 flasks or at $3x10^5$cells/well in 6 well plates for protein and RNA isolation respectively. The following day, medium was replaced with fresh, untreated medium or medium containing 0.1 μg/mL Anisomycin. Cells were incubated at $37^oC/CO_2$ and RNA and whole cell lysate was collected 24 hours and 48 hours post-treatment for qRT-PCR and western blot experiments.

To determine the role of FOXP3 and FOXP3-induced miRs in the cellular response to Anisomycin, MCF10a cells were transfected with FOXP3 siRNAs, pre-miRs or PNAs prior to Anisomycin treatment. Cells seeded at $3x10^5$ cells/well in 6 well plates were transfected with siRNAs, miRs or PNAs using the HiPerfect transfection reagent as described in Section 3.3.3. After overnight incubation, medium was removed and replaced with fresh medium or medium containing 0.1 μg/mL Anisomycin. Total RNA and whole cell lysate were harvested 24 hours and 48 hours after Anisomycin treatment for subsequent qRT-PCR and western blot experiments. Cells were also harvested for cell viability assays as described in section 5.3.4.

5.3.4 Cell viability assays

Flow cytometric analysis with the BD PE Annexin V kit (BD Biosciences, NJ USA) was used to determine cell viability and detect apoptotic cells in untreated and anisomycin-treated populations. Cell staining and analysis was performed as recommended by the kit instructions. Briefly, Anisomycin and control-treated MCF10a cells were harvested and resuspended at $1x10^6$ cells/mL. Cells were washed twice with cold PBS, before resuspending in 1X Annexin V binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$). Aliquots (100 μL) of cell suspension were transferred to FACS tubes, before

addition of Phycoerythrin (PE) conjugated Annexin V (5 μL) and 5 μL of 7-Aminoactinomycin D (7-AAD, BD Biosciences, NJ USA). Cells were then incubated at room temperature for 15 minutes in the dark before analysis using a flow cytometer. Cells were selected initially using a forward scatter/side scatter plot, before gating on an FL2 (Annexin V)/FL3 (7-AAD) plot.

Cell populations were characterised as Annexin V and 7-AAD negative live cell populations, Annexin V single positive early apoptotic cells, Annexin V and 7-AAD double positive late apoptotic cells, and 7-AAD single positive cells that were likely killed by a mechanism independently of apoptosis.

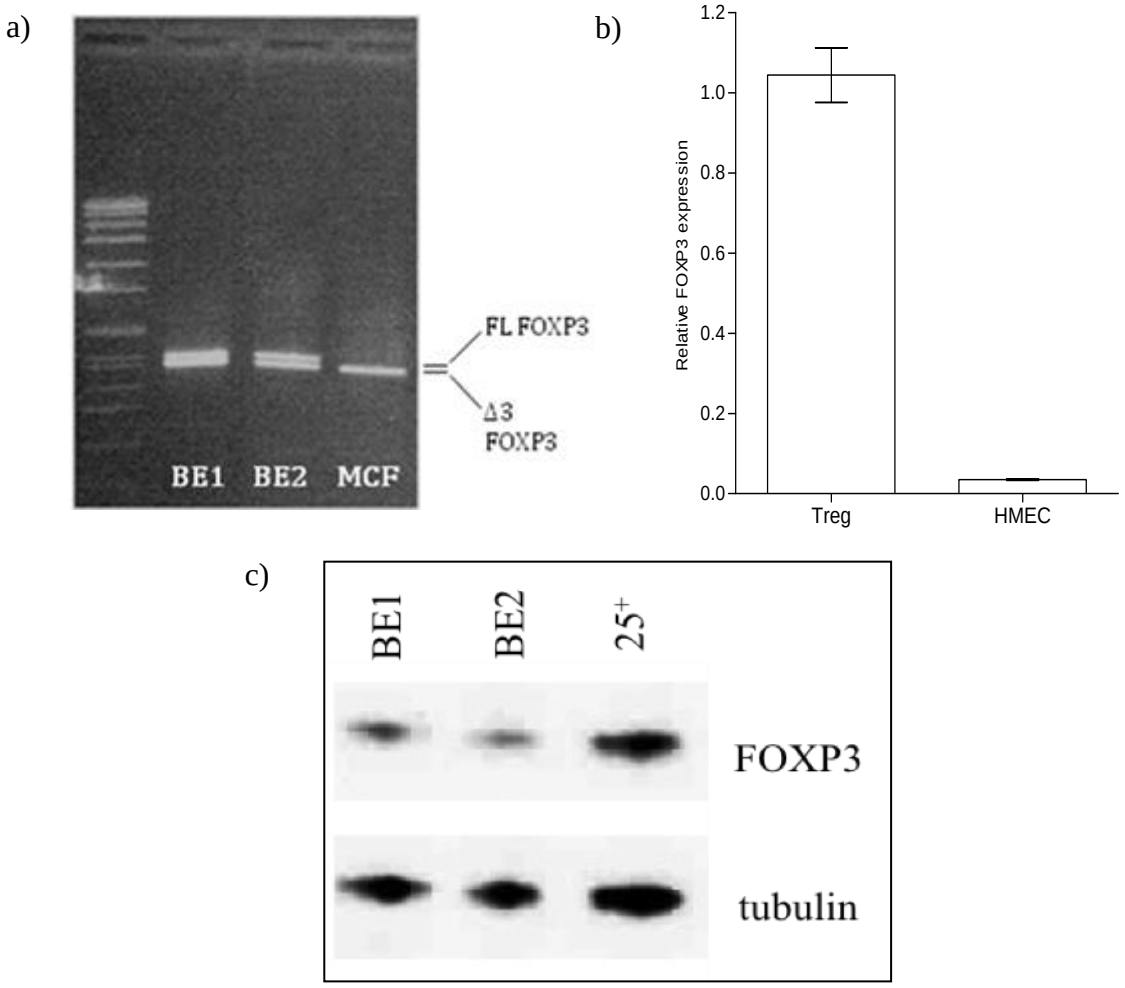
5.4 Results

5.4.1 *FOXP3* is expressed in normal breast epithelial cell lines

To confirm *FOXP3* expression in normal breast epithelia, experiments were performed with primary Human Mammary Epithelial cells (HMEC) isolated from normal breast tissue (Lonza, Switzerland). To detect expression of wildtype *FOXP3* in HMECs, the coding region of *FOXP3* was amplified by PCR from cDNA generated from HMEC total RNA using primers that encompassed the start and termination codons (Table 2.1). Following PCR, the size of PCR products were analysed by agarose gel electrophoresis (Figure 5.1a). The resulting PCR products matched the expected sizes of both the full length (1.3 kb) and the Δ3 (1.2 kb) *FOXP3* isoforms and appeared to be present in approximately equimolar amounts. Further analysis of PCR products via sequencing confirmed that HMECs express the wildtype versions of the two *FOXP3* isoforms (Supplementary Figure 5.S1). In contrast, the immortalised, non-tumorigenic MCF10a breast epithelial cell line expresses only the wildtype Δ3 isoform of *FOXP3* and none of the FL isoform (Supplementary Figure 5.S2). To compare the levels of *FOXP3* transcript expressed in breast epithelia with levels expressed in Treg cells, qRT-PCR was performed. FOXP3 levels were detectable but were significantly lower in breast epithelial cells compared with Treg cells, with at least a 10-fold difference in expression levels observed (Figure 5.1b).

Next, to confirm that FOXP3 protein is also being produced in normal breast epithelia, western blots were performed with whole cell lysate isolated from the HMECs (Figure 5.1c). In HMEC extracts the FOXP3 antibody detected a band of identical molecular weight to that observed in the positive control $CD4^+CD25^+$ Treg extract. Although two

Figure 5.1 ***FOXP3 is expressed in normal breast epithelial cells***



FOXP3 isoform expression in human mammary epithelial cells (HMEC). a) PCR amplification of *FOXP3* in two normal breast epithelial cell lines (BE1 and BE2) and an immortalised cell line (MCF10a). Primers specific for human *FOXP3* (Table 2.1) were used to amplify *FOXP3* isoforms. Gel electrophoresis identified two DNA bands (1.3kb and 1.2kb) that match the expected size of the *FOXP3* isoforms. b) qRT-PCR analysis of relative *FOXP3* mRNA levels in Treg cells compared with HMECs, normalised to expression of the RPL13a reference transcript. n=3. c) Western blot analysis identified FOXP3 protein in whole cell extracts (100 μg) from two cultures of HMECs (BE1 and BE2). Whole cell extract (100 μg) from $CD4^+CD25^+$ Treg cells (25^+) was used as a positive control. Filters were stripped with Western blot stripping solution (Alpha Diagnostics) and an α-tubulin antibody was used as a loading control.

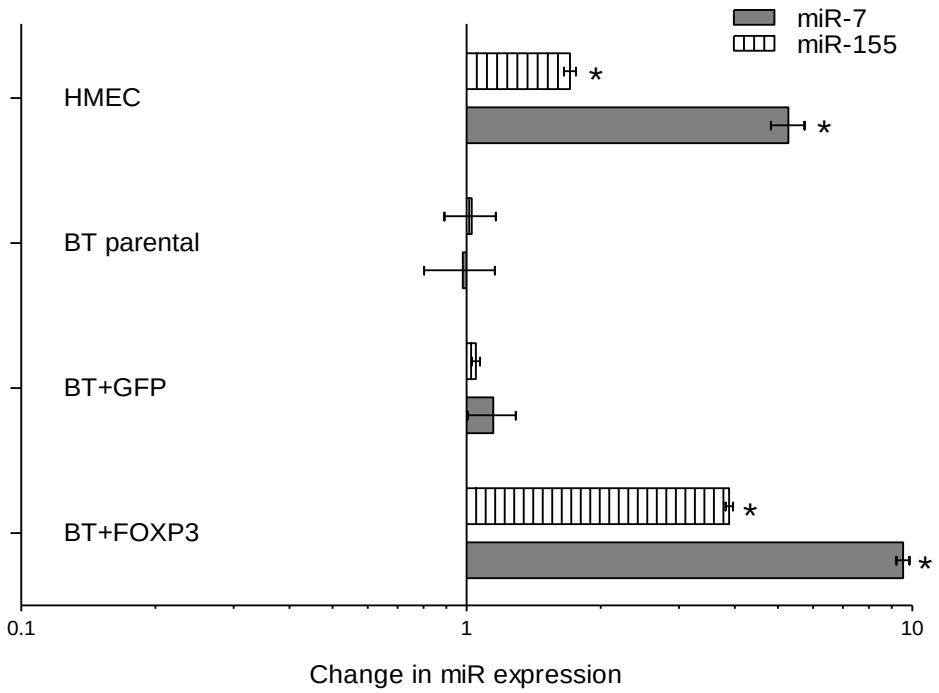
bands were expected, corresponding to the FL and Δ3 isoforms, the presence of a single band could be attributed to a gel resolving issue, with a similar result observed in the $CD4^+CD25^+$ Treg control. Similar to the qRT-PCR results (Figure 5.1b), the levels of FOXP3 protein were considerably lower in HMECs compared with the Treg whole cell lysate. There was no variation in the amount of whole cell lysate loaded, as confirmed by the α-tubulin loading control. Together these data confirm that *FOXP3* is present at both the message and protein level in normal breast epithelial cells.

5.4.2 miR-7 and miR-155 levels are positively regulated by endogenous FOXP3 in normal breast epithelia

To confirm that miR-7 and miR-155 expression levels correlate with *FOXP3* expression levels in HMECs, as seen previously in the breast cancer lines (section 3.4.3), miR expression in the HMEC cell line was determined using miR-specific qRT-PCR and compared with the expression of miR-7 and miR-155 in the BT549 breast cancer cell lines (Figure 5.2a). In addition, to confirm that expression of miR-7 and miR-155 is dependent on FOXP3, endogenous *FOXP3* levels were reduced in the HMEC cell line using a *FOXP3*-specific siRNA, and the resulting levels of miR-7 and miR-155 again determined by miR-specific qRT-PCR (Figure 5.2b). HMECs expressed 6-fold and 2-fold more miR-7 and miR-155 respectively compared with the parental breast cancer cell lines that do not express *FOXP3.* The levels of miR expression observed in the HMECs were similar to those observed in the *FOXP3*-transduced BT549 cells, with both having significantly higher miR expression than the parental and *GFP*-transduced BT549 cell lines ($p<0.0005$). As expected, no significant difference in miR expression was observed in the *GFP*-transduced breast cancer cells compared with the parental cell line. Transfection of the HMECs with *FOXP3*-specific siRNA resulted in a significant reduction in both miR-7 and miR-155 levels, with both showing an approximate 1.4-fold decrease in expression

Figure 5.2 ***MiR-7 and miR-155 are positively regulated by FOXP3 in HMECs***

a)



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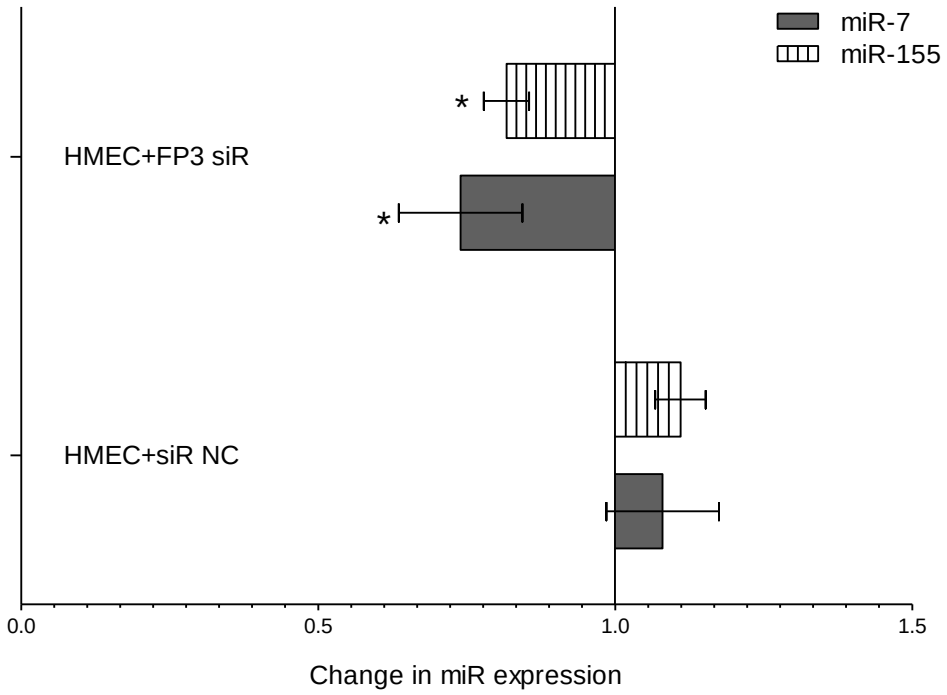

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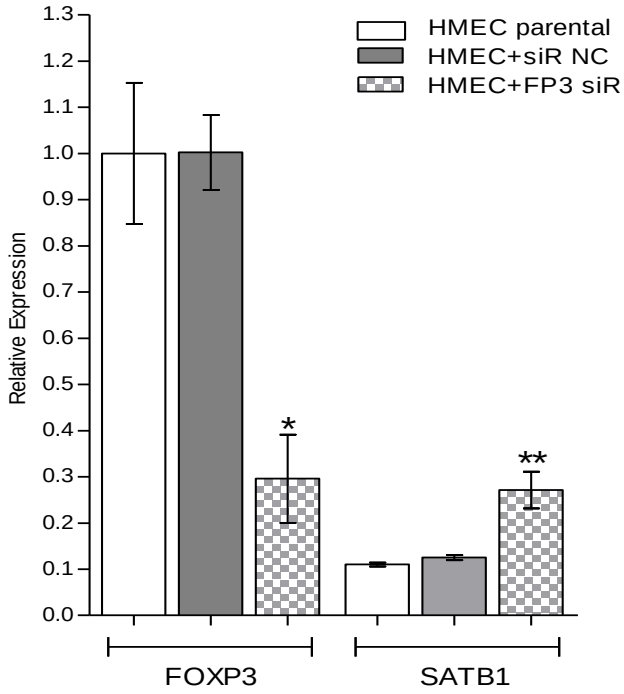


a) miR expression levels in HMEC and *GFP*- and *FOXP3*-transduced BT549s relative to the parental BT549 cell lines by miR-specific RT-PCR. Significantly increased levels of miR-7 (dark bars) and miR-155 (striped bars) were observed in the HMECs and in the *FOXP3*-transduced BT549 cells (*p<0.0005). No significant change in miR levels was seen in the *GFP*-transduced BT549s. b) MiR-specific RT-PCR analysis of miR-7 (dark bars) and miR-155 levels (striped bars) in HMECs transfected with *FOXP3* siRNA relative to parental HMECs. Data from 3 independent experiments, *p<0.02. c) Quantitative RT-PCR analysis of *FOXP3* and *SATB1* message in untreated HMECs (white bars), HMECs treated with a control siRNA (grey bars) or siRNA targeting *FOXP3* (checked bars). Treatment with the *FOXP3* siRNA resulted in a significant reduction in *FOXP3* levels (*p<0.002) and a significant increase in *SATB1* levels (**p<0.01). n=3.

($p<0.02$). No significant difference was observed when comparing the siRNA negative control-transfected cell line with the parental control. Having demonstrated that FOXP3 supports the expression of miR-7 and miR-155 within HMECs, it was of interest to determine if this level of endogenous *FOXP3* expression (and thus endogenous FOXP3-regulated miRs) is involved in *SATB1* suppression in HMECs. In order to confirm FOXP3-mediated regulation of *SATB1*, endogenous *FOXP3* levels were reduced in the normal breast epithelial cell line using a *FOXP3*-specific siRNA. The resulting levels of *FOXP3* and *SATB1* mRNA were determined by qRT-PCR (Figure 5.2c). As shown in Figure 5.2c, *FOXP3* expression levels inversely correlate with *SATB1* levels. Transfection of the HMECs with a *FOXP3* siRNA that caused a 70% knockdown of *FOXP3* mRNA resulted in a 3-fold increase in *SATB1* levels ($n=3$, $p<0.002$ for *FOXP3* reduction and $p<0.01$for *SATB1* increase). No significant difference in *SATB1* levels was observed in cell lines transfected with a non-targeting siRNA when compared with the parental cell line. Together these data support a model in which endogenous FOXP3 may maintain normal breast epithelial cell status in part through the repression of the pro-cancerous oncogene *SATB1*.

5.4.3 The effect of miR-7 and miR-155 overexpression in HMEC and MCF10a cell lines

Given the evidence that introduction of FOXP3-regulated miRs into the BT549 and MDA-MB-231 breast cancer cell lines resulted in reduced growth and invasive potential, it was important to determine if FOXP3 and FOXP3-regulated miRs are involved in regulating similar processes in the HMEC healthy breast epithelial cell line. In addition, the outcome of FOXP3 and FOXP3-regulated miRs was also explored in the MCF10a immortalised

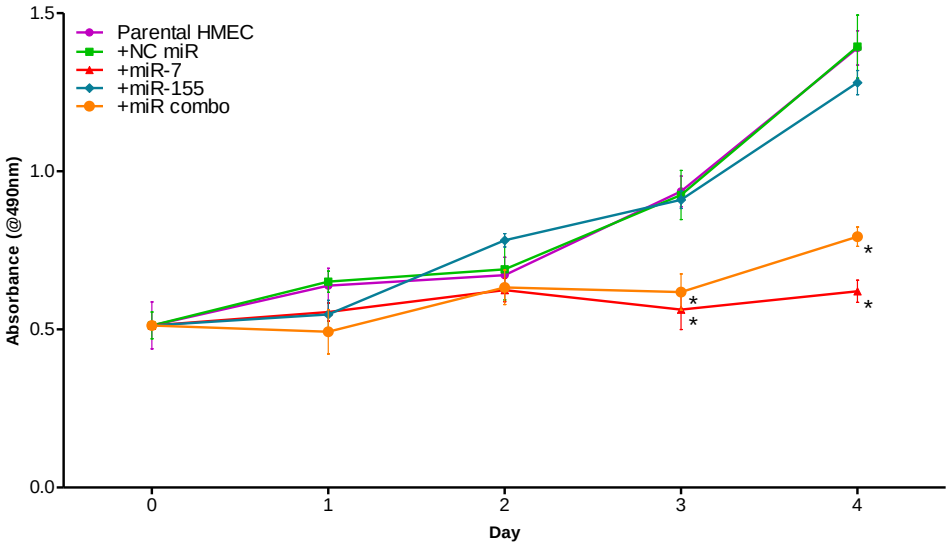
breast epithelial cell line, as it is an appropriate model for breast tumour progression studies (Zientek-Targosz et al., 2008).

To test whether either of these miRs had a negative effect on the growth of HMEC and MCF10a cells, cells were transfected with either pre-miR-7 (10 μM), pre-miR-155 (10 μM) or a combination of both pre-miRs (10 μM consisting of 5 μM of each miR), and the subsequent proliferative activity measured over a 5 day period using the CellTiter 96 Aqueous One Solution Proliferation assay (Promega, WI USA) (Figure 5.3a & b). In both HMEC and MCF10a cells, no significant difference was observed when cells were transfected with either pre-miR-155 or the negative control pre-miR. However, when cells were transfected with pre-miR-7, a significant reduction in proliferative activity was observed from day two of the assay in MCF10a cells and day three in the HMECs ($p<0.05$). In particular, over the course of the assay, no significant increase in proliferative activity as measured by Formazan production was observed in miR-7 treated when compared with day zero. A significant reduction in proliferative activity was also observed in both cell lines when transfected with the combination of pre-miRs ($p=0.05$), however this effect was not greater than that seen when miR-7 is added alone, suggesting that miR-155 does not affect proliferation at the dose tested in this assay.

To determine if miR-7 levels dictate the proliferative activity of HMEC and MCF10a cells, HMEC and MCF10a cells were transfected with PNA miR inhibitors for miR-7 and miR-155. The specificity of the PNA inhibitors was confirmed by miR-specific qRT-PCR (Figure 5.3c), comparing PNA-transfected HMECs with parental cells. Transfection with

Figure 5.3 ***Proliferation is significantly reduced by miR-7***

a)



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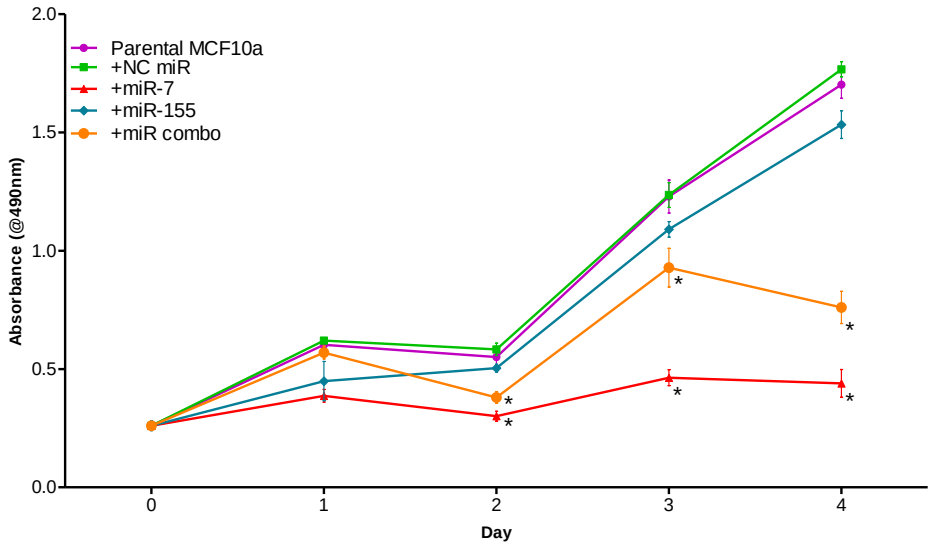

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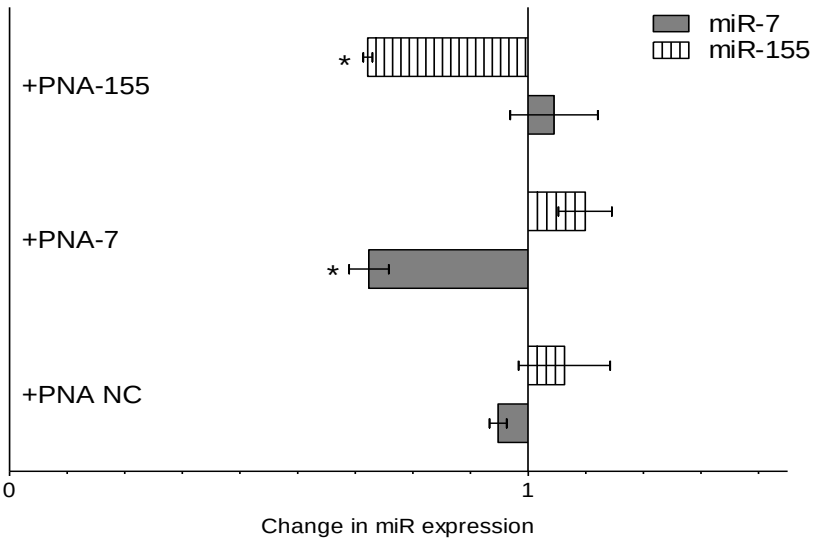

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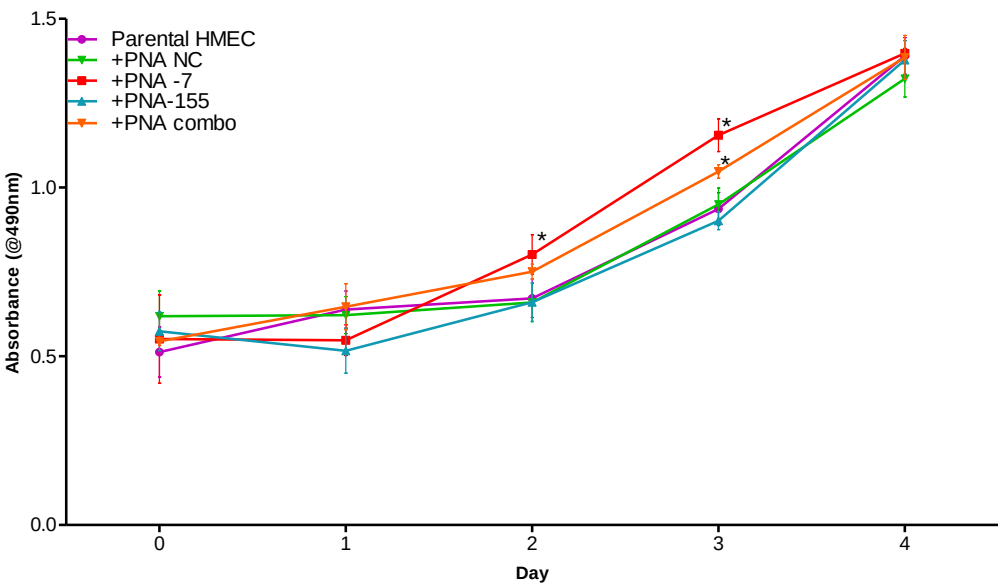
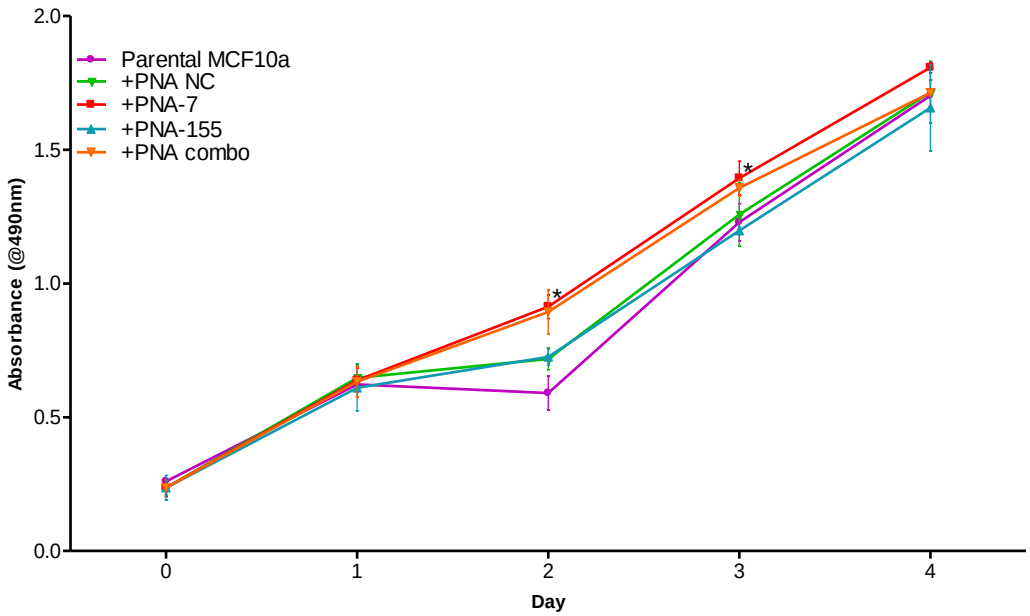


e)

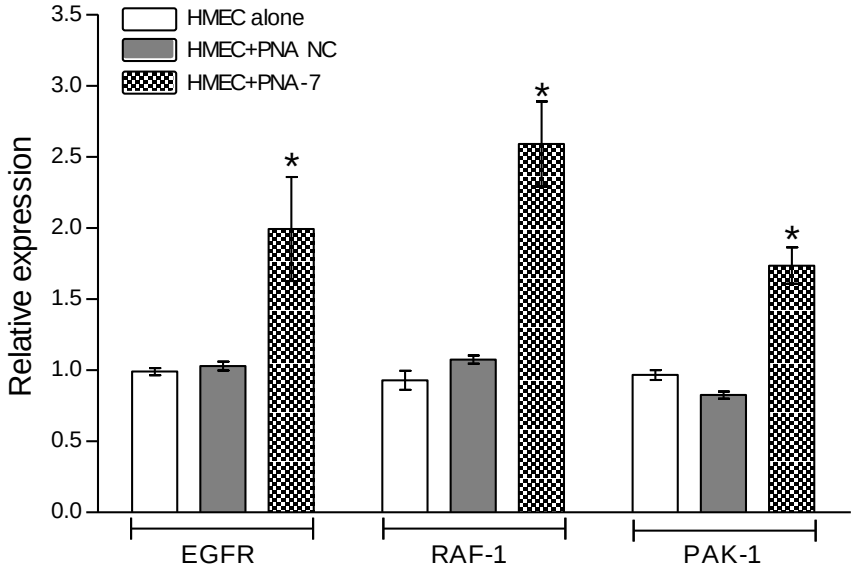


Proliferation of HMEC (a) and MCF10a (b) cell lines transfected with pre-miR-7 (red), pre-miR-155 (blue) or a combination of both pre-miRs (orange) were compared with parental (purple) and control (green) miR-transfected cells using the CellTiter 96 AQueous assay (top graph). c) miR-specific qRT-PCR of miR-7 (dark bars) and miR-155 (striped bars) in HMECs transfected with PNAs, n=3 *p<0.02. Proliferation of HMEC (d) and MCF10a (e) cells transfected with PNA miR inhibitors against miR-7 (red), miR-155 (blue) or both miRs (orange) compared with parental cell lines (purple) and cells transfected with a non-specific miR (green). N=3, *p<0.05.

the PNA inhibitors resulted in miR-specific knockdown, with a 2-fold decrease in miR-7 and miR- 155 seen when treated with PNA-7 and PNA-155 respectively ($n=3$, $p<0.02$). No significant difference in miR expression was observed in the negative control PNA-transfected cells when compared with the parental cells. Consistent with miR-7 playing an important role in controlling the proliferative activity of HMEC and MCF10a cell lines, transfection with a PNA-miR inhibitor for miR-7 resulted in a significant increase in proliferative activity (Figure 5.3d & e, $p<0.05$). As observed with the pre-miR transfections, no change in proliferative activity was detected in cells treated with a miR-155 PNA inhibitor and no additional changes in proliferative activity were detected when a combination of PNA-7 and PNA-155 was used. Together these data indicate that the level of miR-7 expressed in these cells influences the *in vitro* proliferation rate of these cells.

One potential explanation for miR-7 inhibiting the proliferative activity of these cells is that over-expression of miR-7 results in a significant reduction in the levels of miR-7 targets involved in the EGFR pathway. The *in vitro* growth of MCF10a and HMECs is dependent on Epidermal Growth Factor (EGF), and as such, overexpression of miR-7, through its ability to downregulate *EGFR, RAF-1* and *PAK-1*, may impact the ability of cells to respond to this growth factor. To test this theory, qRT-PCR analysis of *EGFR*, *RAF-1* and *PAK-1* message levels were performed in HMECs transfected with a PNA against endogenous miR-7 (PNA-7) and compared with those transfected with a PNA negative control (Figure 5.4). Transfection of HMECs with PNA-7 resulted in at least a 2-fold increase in the levels of all three of these targets ($p<0.002$), suggesting that this miR is involved in the regulation of these EGFR pathway targets.

Figure 5.4 ***miR-7 targets EGFR pathway genes in HMECs***



Quantitative RT-PCR analysis of known miR-7 targets in untreated HMECs (white bars) and HMECs treated with a negative control PNA (grey bars) or a PNA targeting miR-7 (checked bars) (*$p<0.002$, n=3). HMECs were transfected with 100nM PNA for 24 hours before harvesting total RNA for cDNA production and subsequent qRT-PCR.

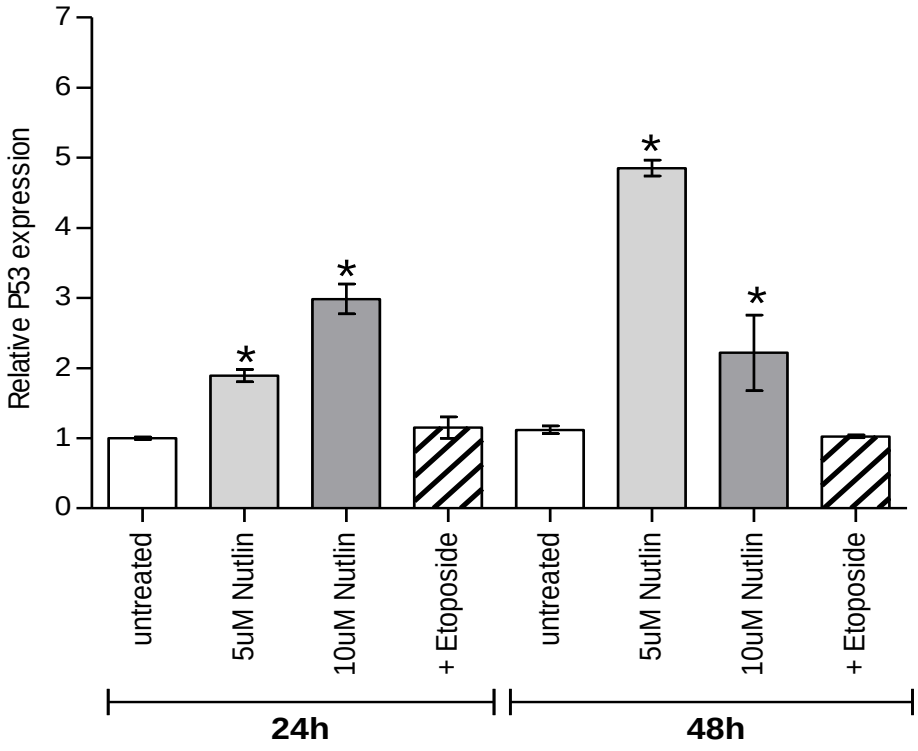
5.4.4 p53 expression does not influence FOXP3 levels in MCF10a cells

In previous studies, treatment of breast cancer cell lines with genotoxic agents Etoposide and Nutlin-3a has been shown to result in the induction of *FOXP3* in a p53-dependent manner (Jung et al., 2010). As miR-7 is regulated by FOXP3, and results in a significant reduction in proliferative activity, it was hypothesised that FOXP3-regulated miR-7 may therefore play an important role in the DNA damage response. To test this hypothesis, MCF10a cells were treated with Nutlin-3a and Etoposide (as described in Section 5.3.2), and subsequent expression levels of *p53* and *FOXP3* were examined. MCF10a cells were treated with either Nutlin-3a, (5 μM or 10 μM) or Etoposide (20 μM) as recommended by published work (Jung et al., 2010) and subsequent levels of *p53* message and protein determined 24 hours and 48 hours post-treatment by qRT-PCR (Figure 5.5a) and western blot (Figure 5.5b) respectively. Message levels of p53 were significantly increased in the Nutlin-3a-treated MCF10a cells at both concentrations. Consistent with the observed changes in the message levels of *p53*, western blots of MCF10a cells treated with Nutlin-3a showed increased p53 protein levels. Unexpectedly, no increase in p53 protein was seen in the Etoposide-treated cells at either 24 hour or 48 hour time points.

As *p53* levels are increased upon Nutlin-3a treatment of MCF10a cells, qRT-PCR analysis was performed to determine if *FOXP3* expression was induced as a result of Nutlin-3a treatment (Figure 5.5c). Results indicated that *FOXP3* expression levels were not increased upon induction of *p53*, with no changes in *FOXP3* expression levels observed at both the 24 hour and 48 hour time points at either concentration of Nutlin-3a treatment. This suggests that in the MCF10a cell line, *p53* induction does not result in the upregulation of *FOXP3* expression. Further investigations into the role of FOXP3 in p53 responses were therefore not pursued.

Figure 5.5 ***Nutlin-3a and Etoposide treatment does not result in elevated FOXP3 levels***

a)



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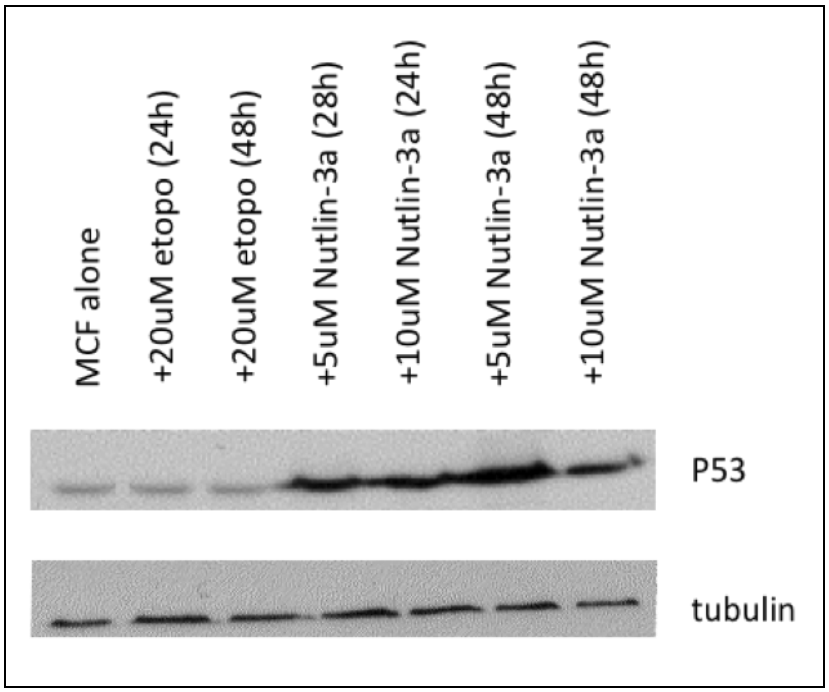
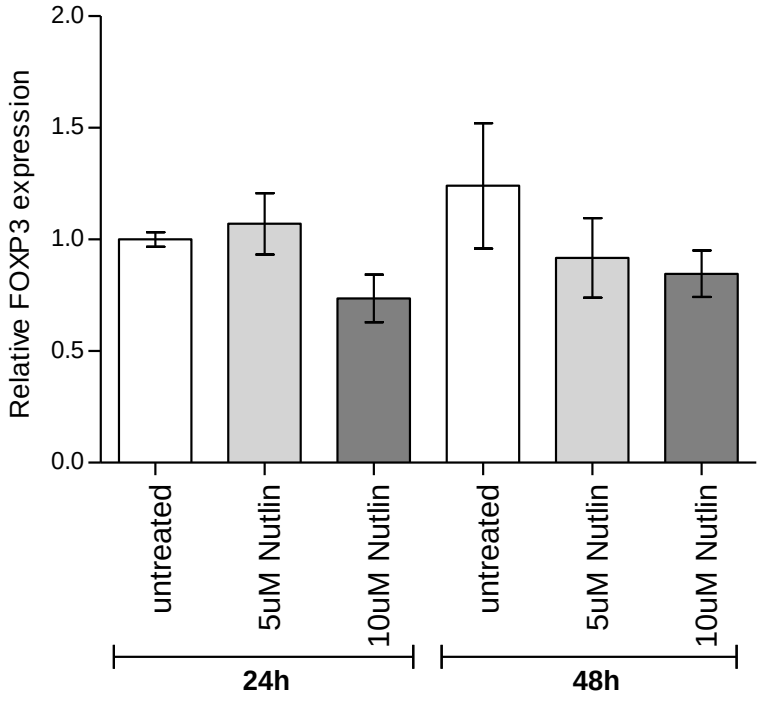


c)



FOXP3 levels are not elevated by Nutlin-3a and Etoposide treatment. a) *p53* mRNA levels in the MCF10a cells treated for 24 hours or 48 hours with Nutlin-3a (grey bars) or Etoposide (black bars) as determined by qRT-PCR (n=3, *p<0.05). b) p53 protein induction is detected in Nutlin-3a-treated MCF10a cells, but is not induced in cells treated with Etoposide as determined by western blotting. The loading control is α-tubulin (bottom row). c) *FOXP3* mRNA levels in the MCF10a cells treated with two concentrations of Nutlin-3a (grey bars) for 24 hours or 48 hours as determined by qRT-PCR (n=3).

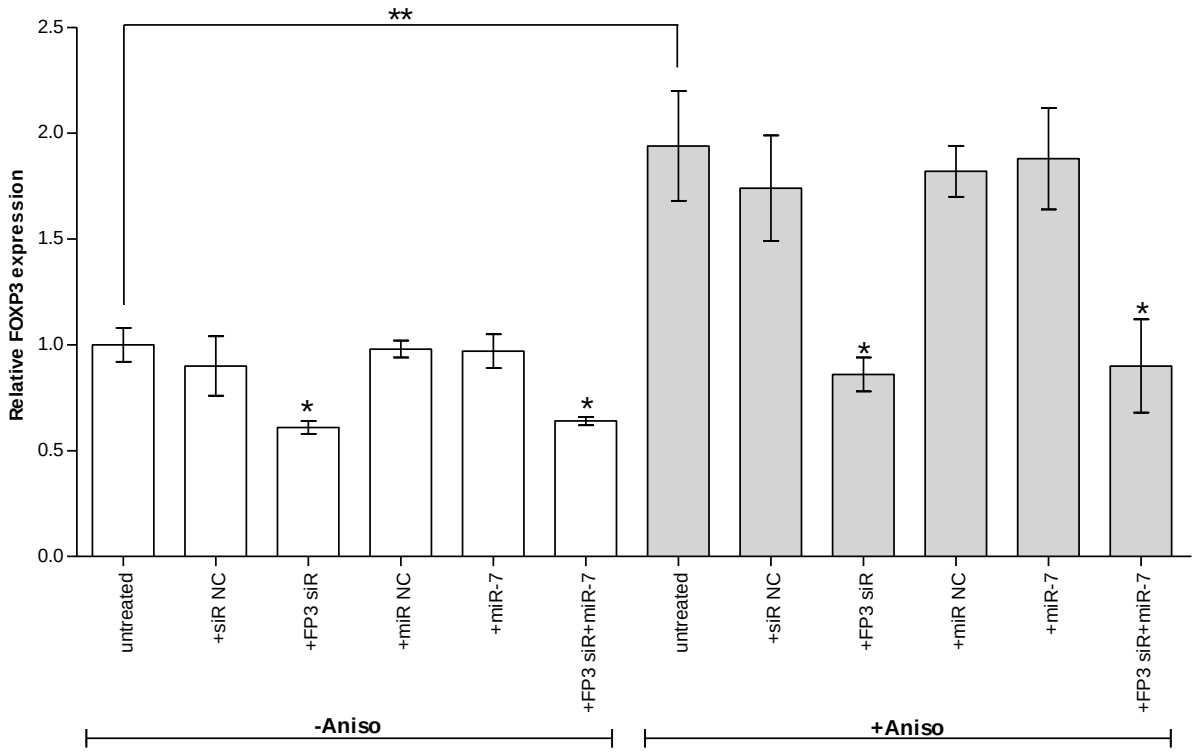
5.4.5 Re-activation of FOXP3 by Anisomycin treatment

Anisomycin treatment of normal and breast cancer cell lines has indicated that *FOXP3* expression may be regulated by the activation and binding of a heterodimer of activating transcription factor 2 (ATF-2) and c-Jun to a conserved enhancer region within intron 1 of the human and mouse *FOXP3* gene (Liu et al., 2009b). In previous studies, the Anisomycin-induced effects on breast cancer growth were associated with its ability to induce *FOXP3* (Liu et al., 2009b), and as such it was of interest to determine if miR-7 induction plays a role in this process.

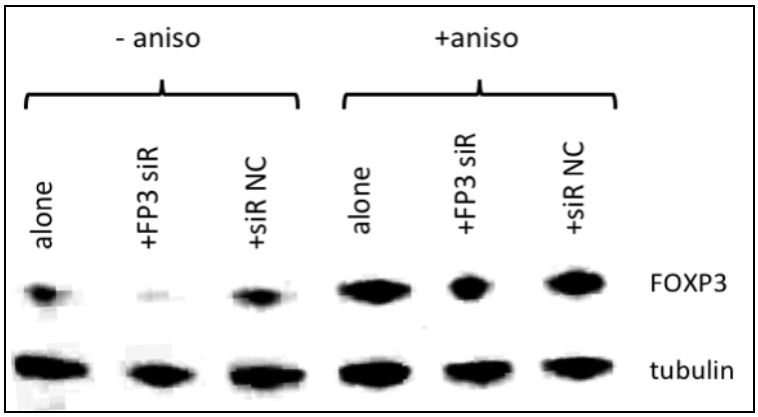
To confirm that Anisomycin treatment results in upregulation of *FOXP3*, qRT-PCR analysis was performed on untreated and Anisomycin-treated MCF10a cells (Figure 5.6a). In addition, a subset of these cells were transfected with a siRNA negative control, *FOXP3*-specific siRNA, a pre-miR negative control, pre-miR-7 or a combination of *FOXP3* siRNA and pre-miR-7, prior to Anisomycin treatment. Treatment with Anisomycin resulted in a significant increase in *FOXP3* expression (2-fold increase, $^{**}p<0.02$), which was lost when cells were first transfected with a *FOXP3*-specific siRNA ($^{*}p<0.05$). No significant difference in *FOXP3* levels was observed in either control siRNA-transfected cell lines when compared with the un-transfected controls or in miR-7 transfected cells when compared with any of the controls. In addition, no change was observed in *FOXP3* levels in MCF10 cells co-transfected with both miR-7 and *FOXP3*-siRNA when compared with *FOXP3*-siRNA alone, indicating that increased miR-7 levels do not influence *FOXP3* expression. To confirm that induction of *FOXP3* expression by Anisomycin seen at the message level translated to increased FOXP3 protein levels, western blot analysis was performed on untreated MCF10a cells compared with cells treated with Anisomycin

Figure 5.6 ***Anisomycin treatment results in upregulated FOXP3 expression***

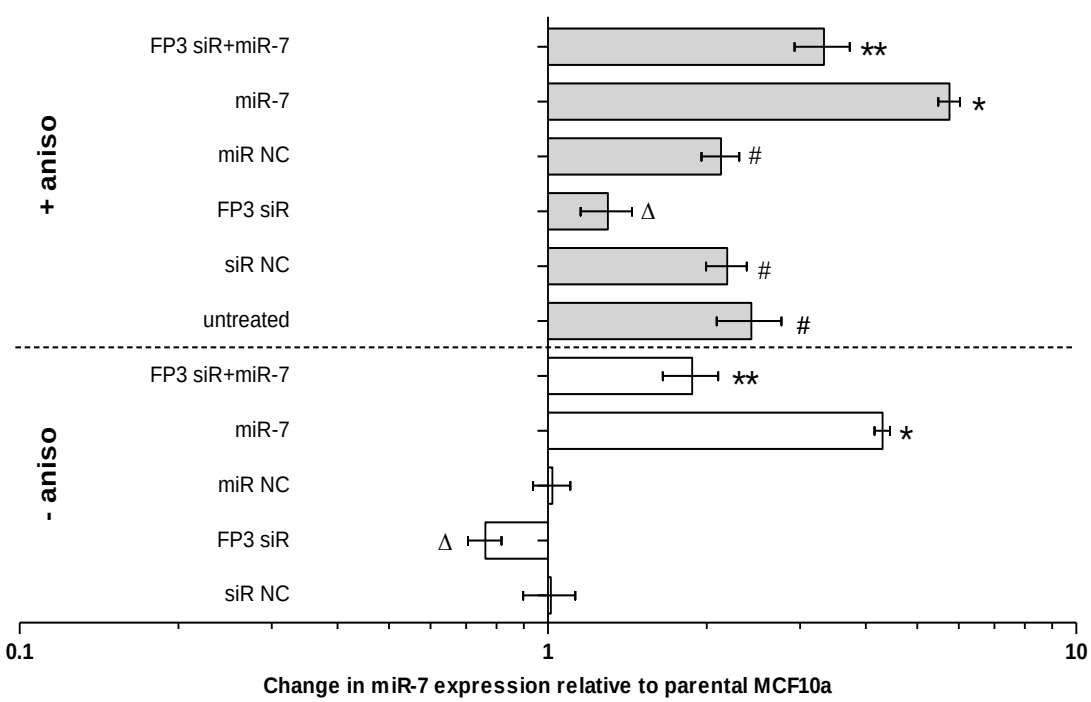
a)



b)



c)



a) *FOXP3* mRNA levels in untreated MCF10a cells (white bars) compared with Anisomycin-treated MCF10a cells (grey bars) as determined by qRT-PCR. Pre-miR and siRNA pre-treatments are indicated on the X axis. Significant reduction compared with controls =*$p<0.05$, significant increase compared with controls =**$p<0.02$, n=3. b) FOXP3 protein expression in untreated MCF10a cells or cells treated with Anisomycin as determined by western blot. Cells were transfected with a siRNA negative control (siR NC) or a *FOXP3*-specific siRNA (FP3 siR). Western blot filters were stripped and re-probed with α-tubulin as a loading control. Figure is representative of 3 independent experiments. c) miR-7 expression levels in untreated MCF10a cells (white bars) compared with MCF10a cells treated with Anisomycin (grey bars) as determined relative to the parental, untreated MCF10a cell line by miR-specific RT-PCR. Significant downregulation compared with siRNA NC = Δ, $p<0.007$; significant upregulation compared with – aniso = #, $p<2.2x10^{-6}$; significant upregulation compared with pre-miR negative control (miR NC) = *, $p<8.5x10^{-11}$; significant downregulation compared with miR-7 = **, $p<2.2x10^{-7}$. n=3.

(Figure 5.6b). FOXP3 protein levels were higher in cells treated with Anisomycin, with a reduction in FOXP3 levels observed when cells were pre-treated with a *FOXP3*-specific siRNA. The level of FOXP3 protein in the siRNA negative control-transfected MCF10a cells was not significantly different from the levels seen in un-transfected controls. Together these data confirmed that Anisomycin treatment of MCF10a cells results in the upregulated expression of *FOXP3*.

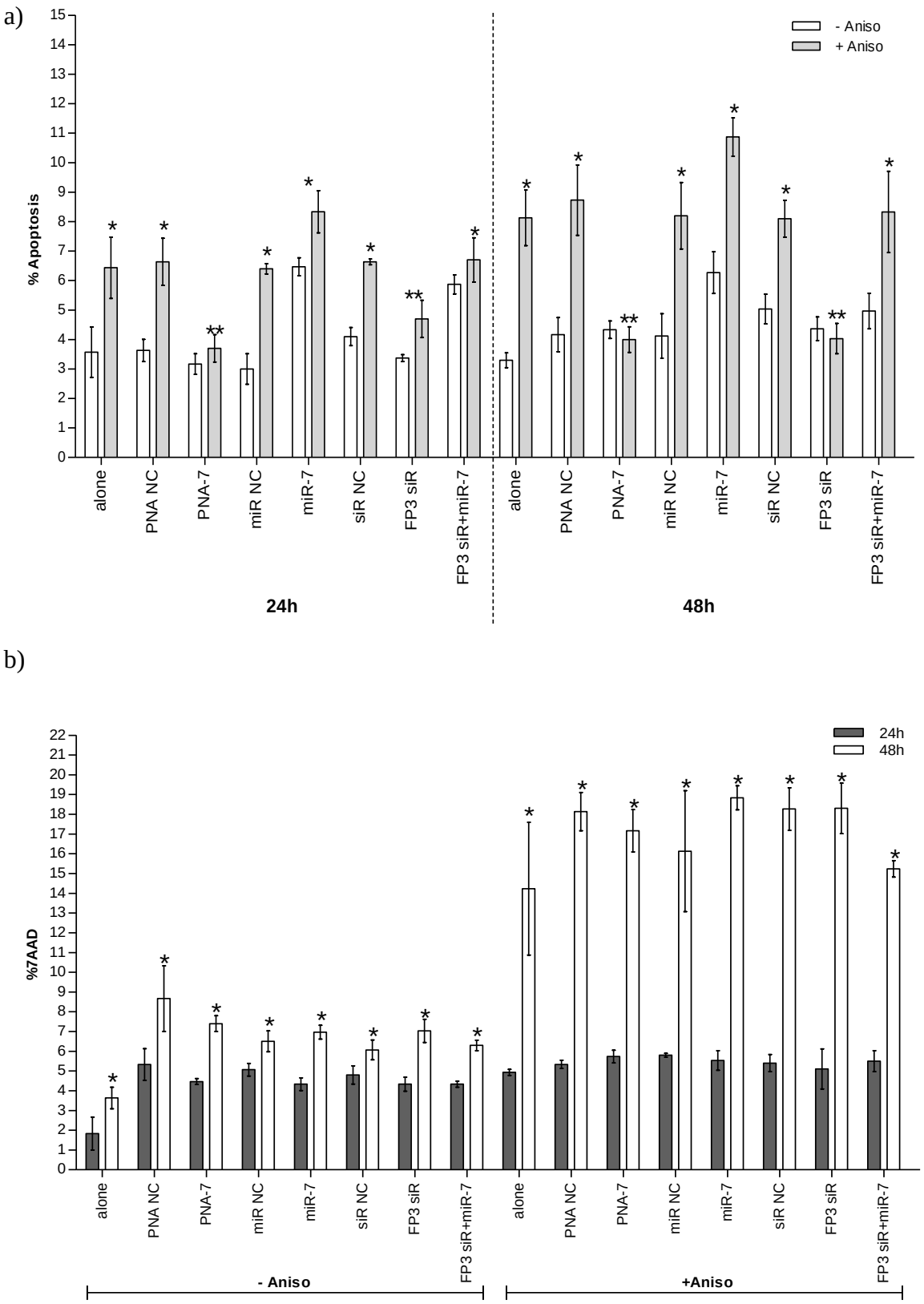
To confirm the link between *FOXP3* expression and the expression of miR-7 in Anisomycin-treated cells, miR-specific RT-PCR was performed (Figure 5.6c). Treatment with Anisomycin resulted in a significant 2-fold increase in miR-7 expression when compared with untreated MCF10a cells (#$p<2.2x10^{-6}$). This upregulation of miR-7 was significantly reduced in cells pre- transfected with a *FOXP3*-specific siRNA ($\Delta p<0.007$), suggesting that the Anisomycin upregulation of miR-7 occurs via *FOXP3* induction. MiR-7 levels were further increased in cells pre-transfected with pre-miR-7 (*$p<8.5x10^{-11}$), which was partially reduced when co-transfected with the *FOXP3*-specific siRNA (**$p<2.2x10^{-7}$). In MCF10a cells that were not treated with Anisomycin, miR-7 levels were significantly reduced when cells were transfected with a *FOXP3*-specific siRNA (2-fold decrease, $\Delta p<0.007$), but significant upregulation was observed when cells were transfected with a pre-miR-7 construct (4-fold increase, *$p<8.5x10^{-11}$). However, this pre-miR-7-induced upregulation was partially reduced upon co-transfection with *FOXP3*-siRNA (**$p<2.2x10^{-7}$). Together these results demonstrate that FOXP3 is important for the regulation of miR-7, and that treatment with Anisomycin results in significant upregulation of both *FOXP3* and miR-7.

5.4.6 Influence of Anisomycin and *FOXP3* activation on cell viability

Previous work has reported that Anisomycin reactivates the expression of *FOXP3*, leading to the induction of apoptosis in two breast cancer cell lines (Liu et al., 2009b). As *FOXP3* reactivation via Anisomycin is reported to induce apoptosis, it was hypothesised that this may be in part due to FOXP3-mediated upregulation of miR-7 expression levels.

To determine if *FOXP3* and miR-7 levels influence apoptosis, Annexin V and 7-AAD staining was performed in MCF10a cells treated with Anisomycin, pre-miR-7, PNA-7 and *FOXP3*-specific siRNA (Figure 5.7). Annexin V binds to phosphatidylserine (PS) that has translocated to the external cellular membrane as a result of apoptosis. Treatment of MCF10a cells with Anisomycin doubled the percentage of Annexin V-positive cells undergoing apoptosis when compared with untreated cells at both 24 hours and 48 hours after treatment ($p<0.005$, Figure 5.7a). Blocking endogenous miR-7 or *FOXP3* by treatment with a *FOXP3*-specific siRNA or PNA miR-7 inhibitor (PNA-7) alone almost completely abolished the anisomycin-induced increase in apoptosis ($p<0.003$). Interestingly, particularly at the 24 hour time point, miR-7 transfection of untreated MCF10a cells was as effective as Anisomycin treatment at inducing apoptosis. Also, miR-7 transfection of MCF10a cells treated with Anisomycin gave a slightly higher rate of Annexin V-positive cells when compared with those treated with Anisomycin alone. When cells were transfected with both *FOXP3* siRNA and pre-miR-7 before treating with Anisomycin, the levels of Annexin V were similar to those observed in cells transfected with miR-7 alone and in Anisomycin-treated control cells. This suggests that FOXP3-induced apoptosis is dependent on alterations in miR-7 levels.

Figure 5.7 ***FOXP3 and miR-7 influence apoptosis***



a) The percentage of cells that stained with AnnexinV in untreated MCF10a cells (white bars) and MCF10a cells treated with Anisomycin (grey bars), as determined by flow cytometry. Cells were transfected with a pre-miR negative control (miR NC), pre-miR-7 (miR-7), siRNA negative control (siR NC), *FOXP3*-specific siRNA (FP3 siR), PNA negative control (PNA NC), a PNA targeting miR-7 (PNA-7), or a combination of *FOXP3* siRNA and pre-miR-7. Cells were analysed 24 hours and 48 hours after treatment with Anisomycin. Anisomycin treatment caused a significant increase in apoptosis when compared with untreated controls (*$p<0.005$). A significant reduction in levels of apoptosis was observed when cells were treated with PNA-7 and *FOXP3* siRNA (**$p<0.002$). b) The percentage of cells that stained with 7-AAD in untreated MCF10a cells and cells treated with Anisomycin, as determined by flow cytometry. Cells were treated as described above, and analysed 24 hours and 48 hours after Anisomycin treatment. A significant increase in cell death was observed when cells were treated with Anisomycin (*$p<0.05$). n=4.

During the time course of Anisomycin treatment in these experiments, a general decrease in cell viability was observed, with increased Anisomycin concentration or extended time course leading to substantial cell death. This suggests that Anisomycin may have a more general effect on cell viability independently of miR-7 or *FOXP3* induction alone. Therefore, it was of interest to determine if the reason for this was toxicity associated with the treatment with Anisomycin or the transfection process. Therefore untreated and Anisomycin-treated cells were also stained with 7-AAD, a fluorescent chemical compound with a strong affinity for DNA. This compound does not readily pass through the membrane, meaning that cells with compromised membranes stain with 7-AAD while live cells with intact membranes do not. The resulting proportion of 7-AAD-positive cells was then determined by flow cytometric analysis (Figure 5.7b). In the absence of Anisomycin treatment, a 2% to 3% increase in cell death was observed at both 24 and 48 hours in all transfected cells compared with the untransfected parental control, suggesting that the transfection process has a small effect on cell viability. Anisomycin treatment alone for 24 hours resulted in an approximate 2% increase in the percentage of dead cells compared with untreated cells. This level of cell death detected did not significantly alter in transfected cells treated with Anisomycin. However at the 48 hour time point, under all conditions, Anisomycin caused much greater cell death than those that were not treated with Anisomycin, with the percentage of dead cells increasing 3-fold to approximately 15% to18% of the total population, compared with approximately 5% to 6% in untreated populations. No significant difference in the level of cell death in siRNA and PNA-transfected cells compared with non-transfected cells was observed, suggesting that this increase was not due to *FOXP3* or miR-7 induction. These results indicate that treatment of the MCF10a cells with Anisomycin, while inducing FOXP3 and miR-7 dependent apoptosis, also results in additional cell death independent of apoptosis and FOXP3 and miR-7 induction.

5.5 Discussion

This chapter investigates the role of FOXP3 in normal primary breast epithelial cells (HMEC) and in an immortalised breast epithelial cell line (MCF10a) that displays a 'near normal' phenotype (Zientek-Targosz et al., 2008). This has allowed insight into the potential role of *FOXP3* and two microRNAs, miR-7 and miR-155, in the maintenance of normal breast epithelial cell status, and suggests additional mechanisms by which *FOXP3* loss may contribute to the progression of breast cancer.

It was hypothesised that FOXP3-regulated miRs form part of FOXP3 tumour suppressive activity. Although *FOXP3* expression in non-T cell populations has been controversial (Fontenot et al., 2003, Kim et al., 2009), accumulating evidence, including conditional gene targeting, immunohistochemistry and expression profiling, has now demonstrated that *FOXP3* is expressed in healthy epithelial cells from several tissues (Wang et al., 2009, Zuo et al., 2007b, Zhang and Sun, 2010), and has linked its presence or absence in normal and cancerous cells with disease outcomes. For example, recent work investigating the prognostic significance of FOXP3 expression in $HER2^+$ breast cancer cells found that FOXP3 is linked with a better prognostic outcome in patients treated with neo-adjuvant chemotherapy (Ladoire et al., 2011). Furthermore, FOXP3 expression in breast cancer cells has also been independently associated with improved overall survival in patients treated with anthracycline-based adjuvant chemotherapy (Ladoire et al., 2012). In this thesis, both FOXP3 message and protein were detectable in the normal HMEC cell line, with HMECs expressing two isoforms (the full length isoform and the Δ3 isoform), also expressed in human Treg cells. This supports the hypothesis of this thesis, with confirmation that *FOXP3* expression is present in normal breast epithelial cells (Section 5.4.1) but is lost in breast cancer cell lines (Section 3.4.1). Compared with cord blood $CD4^+CD25^+$ T cells, a

population highly enriched for T regulatory cells, *FOXP3* expression in cultured human breast epithelial cells was found to be relatively low but not absent. For example in this thesis, qRT-PCR and western blot analysis of mRNA and protein levels of FOXP3 in the $CD4^{+}CD25^{+}$ Treg cells compared with the HMECs, showed clearly detectable but significantly lower levels of FOXP3 in the HMECs. This is consistent with mouse studies, which also reported a much lower level of FOXP3 in breast epithelial tissue compared with Treg cells (Chen et al., 2008b). This low level of *FOXP3* expression may therefore explain the contradictory reports regarding *FOXP3* expression in epithelia. For example, in mouse studies reporting a lack of FOXP3 in epithelia, mice either co-expressing GFP (Liston et al., 2007) or a Diphtheria toxin (DT) receptor from a modified *FOXP3* allele (Kim et al., 2009) were used. The level of GFP reporter in these systems may have been below the sensitivity of GFP detection relative to the auto-fluorescent background, or too little Diphtheria toxin (DT) receptor was co-expressed from the *FOXP3* locus to kill cells following DT administration (Kim et al., 2009). In another study performed in similar *FOXP*-GFP mice, an anti-GFP antibody amplification step was required to see *FOXP3* expression in epithelial cells (Chen et al., 2008b), indicating that the low levels of *FOXP3* makes it difficult to detect expression by GFP. Importantly, FOXP3 protein has also been identified in human breast epithelial samples and cells lines by immunohistochemistry (Chen et al., 2008b, Zuo et al., 2007b, Ladoire et al., 2011, Ladoire et al., 2012), with these studies once again reporting that the levels of FOXP3 in these cells are significantly lower than the levels see in Treg cells (Chen et al., 2008b).

As reported previously (Zuo et al., 2007b), investigation of *FOXP3* expression in the immortalised, non-tumorigenic MCF10a cell line found that only the natural splice variant encoding an isoform of *FOXP3* lacking exon 3 is expressed in these cells. Sequencing of

the entire coding region of this splice variant confirmed that wild-type Δ3 *FOXP3* was expressed (Supplementary Figure 5S2). Consistent with previous reports (Zuo et al., 2007b), loss of the FL *FOXP3* isoform was observed in MCF10a cells. Interestingly, other breast cancer cell lines also do not express the full length version of *FOXP3* (Zuo et al., 2007b), suggesting that loss of FL *FOXP3* is a common occurrence. Several reports have now associated non-redundant interactions and activities to the different splice variants of *FOXP3* (Du et al., 2008, Heinze et al., 2011), raising the possibility that FL *FOXP3* has critical, non-redundant functions in breast epithelia. However, careful evaluation of the two different isoforms needs to be undertaken in breast cancer cell lines to investigate this. To date, only cDNA encoding the FL version of *FOXP3* has been used.

In chapter 3 it was demonstrated that re-introduction of a cDNA coding FL *FOXP3* into two highly aggressive breast cancer cell lines results in increased expression of miR-7 and miR-155, two microRNAs that were shown in chapters 3 and 4 to be involved in the inhibition of proliferation and cell invasion in *in vitro* assays. However, these experiments involved expression of a *FOXP3* transgene at supra-physiological levels, leaving open the possibility that the effects observed represent an artefact of this high level of expression. It was thus important to investigate expression and regulation of these miRs in the HMEC cell line (Section 5.4.2), in the absence of forced expression of *FOXP3*. The finding that changes in miR levels positively correlate with *FOXP3* levels strongly support the hypothesis that these miRs are regulated by *FOXP3.* Consistent with this proposal, miR-specific RT-PCR analysis in HMEC and *FOXP3*-expressing breast cancer cells confirmed that the levels of both miR-7 and miR-155 were higher in the HMEC and *FOXP3*-expressing breast cancer cell lines than in the FOXP3-negative parental lines. More importantly, when endogenous *FOXP3* levels in HMECs were decreased with *FOXP3*-

specific siRNA, miR-7 and miR-155 levels were also downregulated. Although miR levels in the HMECs were not as high as the levels observed in the *FOXP3*-transduced breast cancer cell lines, this likely reflects the lower level of endogenous *FOXP3* expression in HMECs compared with that achieved via exogenous expression of the *FOXP3* transgene in *FOXP3*-transduced breast cancer cell lines.

Knockdown of endogenous *FOXP3* resulted in a significant increase in *SATB1* levels in HMECs, confirming that endogenous FOXP3 (and thus FOXP3-regulated miRs) were responsible for the suppression of *SATB1* in normal breast epithelial cells. Although the original paper by Han *et al.* reported no expression of SATB1 mRNA or protein in non-aggressive breast cancer cell lines and normal tissues (Han et al., 2008), other groups have subsequently reported that there are baseline levels of *SATB1* mRNA and protein in normal human breast epithelia samples (Patani et al., 2009) and in normal gastric epithelia (Lu et al., 2010b). Our results showing low levels of *SATB1* at the mRNA level are consistent with these reports, but need to be confirmed at the protein level. Importantly, reduction of *FOXP3* leads to increased *SATB1* expression, indicating that endogenous *SATB1* is a target for endogenous FOXP3 in these cells. In other cell types, the level of *SATB1* has been shown to determine its function. For example, in T cells, low levels of *SATB1* are required for differentiation and viability, but increased levels are required for activation in response to immunological stimuli (Beyer et al., 2011), while in embryonic stem cells, a balance between *SATB1* and *SATB2* determines if cells self-renew or differentiate (Savarese et al., 2009). This raises the possibility that low levels of *SATB1* are required for normal epithelial cell function but levels need to be strictly controlled by FOXP3 and other mechanisms, such as miR-448 (Li et al., 2011a). Therefore, loss of *FOXP3* may contribute to the overexpression of *SATB1* in breast cancer, as increasing genomic instability during

cancer progression could eventually lead to the inactivation of other regulatory mechanisms, such as miR-448, resulting in further increased *SATB1* levels. To address this, further experiments investigating the effect of *SATB1* on HMECs in which both *FOXP3* and miR-448 are inhibited need to be pursued.

Since overexpression of miR-7 and miR-155 was shown in Chapter 4 to reduce the proliferative activity of breast cancer cell lines, the impact of these microRNAs on normal epithelial cell proliferation was also investigated. MiR-7 and miR-155 levels were manipulated in HMEC and MCF10a cells (Section 5.4.3). As seen in BT549 and MDA-MB-231 breast cancer cell lines, while miR-155 overexpression did not significantly affect proliferative activity, knockdown of miR-7 significantly reduced proliferation, with virtually no increase in proliferation as measured by the CellTiter 96 Aqueous assay observed after day 2 of the experiment. This could be partly attributed to an increase in cell death, as visual inspection of the wells 72 hours after treatment showed the majority of the cells to be dying or dead. In addition, the opposite was observed when miR-7 activity was blocked using a PNA miR-7 inhibitor, with proliferative activity significantly increasing. Later experiments investigating apoptosis indicated that miR-7 could contribute to cell death, with an approximate 2-fold increase in the percentage of apoptotic cells detected in miR-7-transfected cells compared with control miR-transfected cells.

One possible explanation for this result is the involvement of miR-7 in the regulation of growth factor signalling pathways, including the epidermal growth factor receptor (EGFR) signalling pathway (Kefas et al., 2008, Webster et al., 2009). The EGFR pathway is involved in the regulation of proliferation, differentiation and development, and members

of this pathway have been shown to be overexpressed in many human cancers (Bianco et al., 2005). This overexpression has been associated with disease progression, resistance to therapy and poor prognosis. Introduction of miR-7 into lung, breast and glioblastoma cell lines results in the downregulation of EGFR message and protein, by the interaction of miR-7 with two predicted target sites within the 3'UTR of *EGFR* (Kefas et al., 2008). Furthermore, other studies have identified additional miR-7 targets that are known to be involved in the EGFR pathway, including *RAF-1* (Webster et al., 2009) and *PAK-1* (Reddy et al., 2008). In addition, miR-7 has been found to target the insulin-like growth factor receptor 1 (IGFR-1) and insulin receptor substrates (IRS) 1 and 2 (Rai et al., 2011, Cochrane et al., 2010). This suggests that increased miR-7 levels in HMEC and MCF10a cells will render these cells less responsive to mitogenic signals, decreasing proliferation and increasing their susceptibility to apoptosis. Given the dependence of both HMEC and MCF10a on EGF supplementation for their *in vitro* growth, overexpression of miR-7 may result in downregulation of *EGFR* pathway members and subsequently reduce proliferative activity as observed. Upregulation of these members of the EGF signalling pathway by a PNA miR-7 inhibitor may also explain the enhanced proliferation rate observed. Downregulation of the EGFR pathway by miR-7 was also supported by qRT-PCR, with knockdown of endogenous miR-7 with a PNA inhibitor resulting in significant upregulation of *EGFR*, *RAF-1* and *PAK-1*, possibly rendering the cells hyper-responsive to EGF and other mitogens present in the culture medium (Section 5.4.3). Detailed growth and survival assays in limiting levels of EGF will be required to address this. Interestingly, earlier work has linked FOXP3 to the suppression of a second EGFR family member, *HER2* (Zuo et al., 2007b). EGF-mediated induction of EGFR and HER-2 causes these receptors to form homo or heterodimers, leading to a variety of downstream signalling that can regulate cell motility, proliferation, apoptosis and the release of proteases and angiogenic factors (Eccles, 2011). Therefore given the evidence that FOXP3 regulates both

HER2, EGFR2 and downstream effector molecules of these receptors, FOXP3 may be a critical mediator of EGFR signalling in breast epithelia.

Another possible explanation for the apparent loss of proliferation in cells overexpressing miR-7 as measured by the CellTiter 96 Aqueous assay is an increased sensitivity to apoptosis. Previous studies have reported that the induction of *FOXP3* occurs due to activation of the p53 DNA damage pathway, with FOXP3 involved in the resulting growth arrest (Jung et al., 2010). Therefore it was hypothesised that as miR-7 is a downstream target of FOXP3, it may also be important for growth arrest mediated by p53. To test this hypothesis, it was first necessary to induce the p53 pathway, which has been reported to occur in the MCF-7 (*p53* wildtype) breast cancer cell line upon treatment with the genotoxic agents Etoposide and Nutlin-3a (Jung et al., 2010).

The results from the p53 experiments performed in this chapter (Section 5.4.4) appear to contradict published data (Jung et al., 2010), in which induction of wildtype p53 in the breast cancer cell line MCF-7 and the colon cancer cell line HCT116 resulted in *FOXP3* induction. In this work, treatment with Etoposide did not induce expression of *p53* nor alter *FOXP3* expression levels in MCF10a cells. This result was unexpected, as other groups have reported p53 induction with Etoposide in MCF10a cells (Hoenerhoff et al., 2009, Kasiappan et al., 2010). One possible explanation for this is that the difference in dose of Etoposide used to treat the MCF10a cells, was insufficient to induce p53. In Jung *et al.* 20 μM of Etoposide was used to treat HCT116 colon cells, and this was the concentration used for this work. Etoposide was not used to treat MCF10a cells in Jung *et al.* In comparison, Hoenerhoff *et al*. and Kasiappan *et al.* used between 40 μM and 100 μM of

Etoposide to induce p53 in MCF10a cells (Hoenerhoff et al., 2009, Kasiappan et al., 2010). Whether lower doses of Etoposide could also induce p53 in this cell line was not reported in these studies.

Although Etoposide treatment failed to induce *p53* in MCF10a cells in this work, Nutlin-3a treatment resulted in a significant increase in *p53* expression. However, increased p53 levels failed to induce the expression of *FOXP3*, suggesting that in the MCF10a cell line, *FOXP3* could not be induced by p53 activity. This difference could be a result of the cell lines used in the studies. Previous work was performed in the MCF-7 breast cancer cell line (Jung et al., 2010), while this work was performed using the MCF10a cell line. Although both of these cell lines express wildtype *p53*, it is possible that cell line-specific differences may prevent the induction of *FOXP3* by p53 in MCF10a cells. Therefore, currently it is not possible to confirm p53 regulation of human *FOXP3*.

Recently, a complex consisting of ATF-2/c-Jun was found to increase *FOXP3* transcription in breast epithelial cells. The drug Anisomycin has been shown to reactivate expression of *FOXP3* in mouse and human breast cancer cell lines 4T1 and MCF-7, through the induction of *ATF-2* and *c-Jun* (Liu et al., 2009b). Consistent with previous studies (Liu et al., 2009b), Anisomycin was originally added to the MCF10a cell line at 4 different concentrations; 0.1, 0.2, 0.5 and 1.0 μg/mL (Section 5.4.5). However, treatment beyond 0.1 μg/mL appeared to be extremely toxic to the cells by day 1 post-treatment. For this reason, all subsequent experiments were performed using an Anisomycin concentration of 0.1 μg/mL. This toxic effect was not reported in the MCF-7 human cell line or 4T1 mouse cell line in earlier studies with Anisomycin (Liu et al., 2009b), suggesting that it may be a cell

line-specific response, with MCF10a being more sensitive Anisomycin. In addition, the dose of Anisomycin used in the *in vivo* studies performed by Liu *et al.* was not reported, so it is possible that the experiments performed in this thesis used a higher, more toxic dose. Almost complete cell death was also observed when Anisomycin-treated MCF10a cells were cultured for more than 48 hours, and for this reason, no experiments were taken beyond this time point. The reactivation of *FOXP3* by Anisomycin has been proposed to be a potential therapeutic for the treatment of cancers (Liu et al., 2009b). The toxicity issues identified in this study indicates that careful investigation of the risk associated with using this agent as a therapeutic need to be undertaken. *In vivo* treatment of mouse mammary tumours with Anisomycin showed a significant reduction in tumour growth when compared with untreated controls; however no mention is made of any side effects (Liu et al., 2009b). RT-PCR analysis of *FOXP3* expression after treatment with Anisomycin confirmed that *FOXP3* mRNA and protein levels were upregulated, therefore supporting previous work (Liu et al., 2009b).

Proliferation assays described in this chapter demonstrated that overexpression of miR-7 results in a significant reduction in MCF10a proliferative activity. Aside from suppression of the EGFR pathway leading to a direct effect on proliferation, suppression by miR-7 is also likely to regulate other signalling. As described earlier, EGF-mediated signalling via *EGFR* is a complex process that can lead to multiple downstream effects, and inhibition of this pathway has also been linked to decreased survival (Eccles, 2011). In addition, miR-7 has been recently shown to target other factors that are involved in receptor signalling, AKT and P13K, which have reported anti-apoptotic functions (Fang et al., 2012). Annexin-V staining was used to investigate the percentage of apoptosis in Anisomycin treated and untreated MCF10a cells (Section 5.4.6). FOXP3-dependent upregulation of miR-7 in

response to Anisomycin treatment was confirmed, with the role of miR-7 in Anisomycin-mediated apoptosis therefore investigated in MCF10a cells. Results indicated that FOXP3, through the upregulation of miR-7, can influence the levels of Anisomycin-induced apoptosis. Anisomycin treatment resulted in a significant increase in apoptosis, but this increase was significantly reduced when either miR-7 was blocked with a PNA miR-7 inhibitor or when *FOXP3* levels were reduced with a *FOXP3*-specific siRNA. This reduction appears to be a result of increased miR-7 levels, and not a direct result of FOXP3, as co-transfection of MCF10a cells with *FOXP3* siRNA and miR-7 results in levels of apoptosis that are not significantly different from those seen when cells were transfected with miR-7 alone. These results suggest that miR-7 is able to induce apoptosis; however the exact mechanism by which this miR induces apoptosis requires further investigation. It would also be of interest to perform cell counts after treatment with Anisomycin, as this would confirm the results observed with Annexin-V.

Overall, the studies in this chapter have reinforced a role for *FOXP3* and miR-7 in controlling growth in normal epithelial cells, potentially by modifying the ability of cells to respond to growth factor signalling.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The general hypothesis of this thesis is that FOXP3 regulates specific microRNAs to exert part of its tumour suppressor function in breast epithelial cells. The studies outlined in this work show that two miRs, miR-7 and miR-155, are controlled by FOXP3 in breast epithelia. FOXP3 induction of miR-7 led to reduced cell growth and increased apoptosis. Previously, miR-7 has been implicated in the negative regulation of growth factor signalling, with targets including several growth factor receptors, such as EGFR and IGFR1, and a number of downstream intracellular effector molecules used by these receptors. This fact, together with the finding that FOXP3 suppresses the transcription of another EGFR family member, HER2 (Zuo et al., 2007b), indicates that the FOXP3-miR-7 network is associated with the suppression or limiting the response of cells to mitogens such as EGF in the breast. These studies have also identified an important, novel role for FOXP3, miR-7 and miR-155 in breast epithelia; suppression of the pro-metastatic oncogene *SATB1*.

When the research presented in this thesis was initiated, the expression of *FOXP3* in non-T cells was still controversial (Christodoulou et al., 2006, Ebert et al., 2008, Fontenot et al., 2003, Kim et al., 2009). However, consistent with previous reports (Zuo et al., 2007a, Zuo et al., 2007b), this work has confirmed that the full length (FL) and Δ3 isoforms of *FOXP3* are expressed in normal breast epithelial cells, while only the Δ3 isoform is expressed in immortalised MCF10a breast epithelial cells and no *FOXP3* is expressed in BT549 and MDA-MB-231 late stage aggressive breast cancer cell lines. This loss of the FL *FOXP3* isoform in MCF10a cells and other breast cancer cell lines raises the possibility that FL *FOXP3* and Δ3 *FOXP3* have non-redundant roles in epithelial cells, with loss of FL *FOXP3* associated with partial loss of tumour suppressor function. Consistent with this, reactivation or forced expression of FL *FOXP3* in breast cancer cell lines either through

Anisomycin induction or expression of a transgene respectively resulted in significant cell death and/or growth inhibition depending on the cell line used.

Whether the different FOXP3 isoforms have different roles in breast epithelia requires further investigation. Alterations in the relative levels of *FOXP3* isoforms may be significant, given that small changes to the interaction between FOXP3 and its transcriptional partners can result in a significant change in cell phenotypes (Chatila and Williams, 2012). Interestingly, the region encompassing exon 3 has been identified as critical for FOXP3 interaction with the Retinoic acid receptor-related orphan receptor (ROR) alpha (RORα) in T regulatory cells. Loss of exon 3 encoded sequences in the Δ3 isoform disrupts this interaction. Increased RORα activity in Treg cells leads to the expression of genes that define the Th17 subset, and therefore the balance of the two isoforms has been proposed to be involved in regulating Treg stability and T cell lineage plasticity. RORα has recently been shown to play a role in different aspects of breast cancer, including local estrogen production (Odawara et al., 2009), increasing ERα transcriptional activity (Dong et al., 2010) and inhibiting tumour invasiveness (Xiong et al., 2012). Changes in the balance of *FOXP3* isoforms in breast epithelia therefore has the potential to alter RORα activity, resulting in changes to breast epithelial cell phenotype.

A novel finding from this thesis is that two microRNAs identified as FOXP3 targets in Treg cells, miR-7 and miR-155, are also regulated by FOXP3 in breast epithelia. MiR-7 and miR-155 are expressed in healthy breast epithelial cell lines but expression is significantly reduced upon downregulation or loss of *FOXP3*. Consistent with this, the relative expression of these miRs in breast cancer cell lines that do not express functional

FOXP3 was low compared with HMECs, and critically when these breast cancer cell lines were transduced with *FOXP3* lentivirus, miR-7 and miR-155 levels were substantially upregulated. The confirmation that miR-7 and miR-155 are regulated by FOXP3 in breast epithelia led to the proposal that aberrant miR expression may be a significant consequence of *FOXP3* loss during cancer progression. Other miRs are potentially regulated by FOXP3 in breast epithelia based upon the data from the Treg ChIP experiments; however these have not been examined in this thesis. The recent FOXP3 ChIP-seq experiments performed in the MCF-7 cell line did not report or annotate FOXP3 binding sites to miR loci (Katoh et al., 2011). This may be due to the annotation method used, as only protein coding genes containing a FOXP3 binding site located in close proximity to the transcription start site (TSS) and showing differential regulation following *FOXP3* induction were reported (Katoh et al., 2011). As such they may have missed intergenic miRs or did not annotate intragenic miRs located within the intronic regions of coding genes. Presently, the raw ChIP-seq dataset has not been publically released, preventing re-analysis of this data for FOXP3 binding regions associated with miR encoded loci. Therefore the extent of overlap between potential miR targets in BC and Treg cells is not known. Large scale identification of differential miR expression using Taqman Low Density Arrays (TLDA, Applied Biosystems, CA USA), or small RNA-seq in HMEC or BC lines with manipulated FOXP3 levels are planned to identify potential miR targets of FOXP3 for follow-up investigations.

There is some controversy surrounding the roles of miR-7 and miR-155 in cancers, and as such it would be necessary to confirm that miR-7 and miR-155 loss is associated with breast cancer. The role of miR-7 in breast cancer appears to be complex and its function may depend upon the tumour subtype. Multiple studies have shown increased miR-7 levels result in reduced growth suppression, reduced invasion and increased apoptosis in breast

cancer cell lines *in vitro* and *in vivo* mouse models (Kong et al., 2012, Fang et al., 2012, Reddy et al., 2008). In addition, miR-7 levels have been reported to be significantly reduced in primary tumour samples compared with normal breast epithelia (Reddy et al., 2008, Kong et al., 2012) with a negative correlation between miR-7 levels and metastatic state (Kong et al., 2012). It has also been demonstrated that miR-7 expression is linked to the expression of the Homeobox D10 (HoxD10) tumour suppressor (Reddy et al., 2008). MiR-7 was shown to be positively regulated by the HoxD10, with loss of HoxD10 linked to increased cancer invasion (Reddy et al., 2008). However, two studies have reported increased miR-7 levels in ER^+ breast cancers, and this increase is associated with higher aggressiveness and tumour grades (Lyng et al., 2012, Foekens et al., 2008). These data raise the possibility that miR-7 function may be modified by ER^+ status of breast cancer, although no experimental data were provided to show that increased miR-7 levels contributed to increased aggressiveness and tumour grades (Foekens et al., 2008, Lyng et al., 2012).

Recently, miR-7 was found to be positively regulated by estrogen, providing an explanation for the higher abundance of miR-7 found in ER^+ breast cancers compared with ER^- cancers (Cochrane et al., 2010). Interestingly, consistent with miR-7 being involved in a tumour suppressor network in the breast, this study found that miR-7 targeted and reduced the expression of Insulin-like Growth Factor 1 Receptor (IGF1R) and Insulin Receptor substrate 2 (IRS-2) in ER^+ MCF-7 cells (Cochrane et al., 2010). IGF1R is often overexpressed in aggressive cancers with poor prognosis. Downregulation of IGFR1 and intracellular signalling molecules such as IRS-2 by miR-7 in MCF-7 cells is consistent with miR-7 maintaining the ability to suppress growth factor signalling in the presence of intact ER signalling. Whether increased miR-7 levels are involved in conferring

aggressiveness to ER^+ tumours, as suggested by profiling studies (Foekens et al., 2008, Lyng et al., 2012), remains to be confirmed by examining miR-7 function in experimental models of aggressiveness in ER^+ cancers.

In addition to miR-7, the work performed for this thesis found that FOXP3 also upregulates miR-155, which has previously been identified as an oncomiR in many cell types, including in breast epithelia (Kong et al., 2010, Mattiske et al., 2012, Wang and Hua, 2012). This raises the question as to why FOXP3 would upregulate a miR with potential oncomiR activity. One possible reason for why FOXP3 would positively regulate miR-155 levels is that healthy breast epithelial cells require a certain level of miR-155 for normal function and that FOXP3 is responsible for maintaining this level. Increased overexpression of miR-155 in breast cancer cell lines following *FOXP3* re-introduction can therefore be viewed as the mimicking of a normal process, but in an aberrant cellular context. For example, miR-155 has been linked to epithelial-to-mesenchymal transition (EMT) in breast cancer, but EMT is also a process that occurs normally in mammary gland development and function (Xiang et al., 2011, Micalizzi et al., 2010). To determine the role of miR-155 in the breast, human studies could be performed using the immortalised cell line MCF10a. Primary breast cells are difficult to use in such studies, as they have a restricted lifespan. Zinc finger (ZF) genome editing is a relatively new technology that allows introduction of mutations into a ZF, causing it to recognise a specific DNA target site (Mani et al., 2005). The ZF is fused with a nuclease, so that once the ZF binds to the target site, the nuclease introduces double stranded breaks to the target DNA (Mani et al., 2005). This technique would allow targeted deletion of miR-155 in the MCF10a cell line. Subsequent experiments investigating growth, proliferation, apoptosis and *in vitro* models of normal breast development could then be performed to investigate the role of miR-155.

Alternatively, using a Cre/LoxP1 gene targeting system, targeted miR-155 knockout in the mammary glands of mice could be performed. This *in vivo* model could be used to determine the consequence of miR-155 loss in normal mammary epithelial cells.

The investigation of the effects of FOXP3, miR-7 and miR-155 on HMECs and breast cancer cell lines resulted in the identification of the pro-metastatic oncogene *SATB1* as an important target for FOXP3 dependent suppression. In addition, the demonstration that FOXP3, through the regulation of miR-7 levels, is important for setting the level of expression of growth factor (GF) receptor pathways, in particular EGFR signalling, links *FOXP3* expression to these pathways for the first time. It also suggests that one of the key functions of FOXP3 in breast epithelial cells is maintaining the balance of growth signals. The work presented in this thesis describes how expression of both *FOXP3* and *FOXP3*-regulated microRNAs in breast cancer cell lines results in the suppression of the pro-metastatic breast cancer oncogene *SATB1*. SATB1 has been implicated in the promotion of epithelial-to-mesenchymal transition and metastasis in late stage, aggressive breast cancers (Han et al., 2008), with high SATB1 message and protein levels associated with poor patient prognosis and multi-drug resistance (Kohwi-Shigematsu et al., 2012, Li et al., 2010, Patani et al., 2009). Interestingly, a study looking at the mechanism behind development of chemotherapy resistance in MCF-7 cells found that a reduction in miR-7 levels was involved in the development of cisplatin chemotherapeutic agent-resistance in these cells (Pogribny et al., 2010). This study found that miR-7 targets and reduces the expression of the multidrug resistance-associated protein 1 (MRP-1), increasing sensitivity to cisplatin by decreasing efflux (Pogribny et al., 2010). Given the finding that miR-7 can also suppress *SATB1*, as described in this thesis, and the proposed role of SATB1 in multi-drug resistance (Li et al., 2010), it is also possible that upregulation of *SATB1* following miR-7

loss contributes to cisplatin resistance. A role for SATB1 overexpression in cancer progression does not appear to be confined to breast cancer, with a correlation between *SATB1* expression and advanced cancers with poor prognosis also observed in gastric, colorectal, liver, cutaneous malignant melanoma and ovarian cancers (Chen et al., 2011, Cheng et al., 2010, Lu et al., 2010b, Meng et al., 2012, Tu et al., 2012, Xiang et al., 2012). The results in this thesis are consistent with *SATB1* having oncogenic potential in breast cancer, with *SATB1* knockdown in breast cancer cell lines resulting in a reduction in cellular invasiveness. This may also underline an important role for miR-155 in epithelial cells, as published work has shown a role for this miR in the prevention of epithelial-to-mesenchymal transition and metastasis (Xiang et al., 2011), processes that are reported to require expression of the *SATB1* oncogene (Han et al., 2008).

A key finding of this thesis was the demonstration that FOXP3 regulates *SATB1* by both binding directly to the promoter region of the gene, while also upregulating miR-7 and miR-155, which target its 3'UTR for further suppression. This transcriptional and post-transcriptional regulation of *SATB1* constitutes a FOXP3-miR-*SATB1* feed-forward regulatory loop. There is growing evidence that miRs and transcription factors participate in feed-back and feed-forward regulatory loops which are critical in the majority of biological processes (Brosh et al., 2008, Re et al., 2009, Tsang et al., 2007, Xie and Cvekl, 2011, Bracken et al., 2008, Harris and Levine, 2005, Li et al., 2011a, Li et al., 2012). Importantly, it has been established that these feed-back and feed-forward regulatory loops significantly enhance the robustness and responsiveness of the gene regulation network (Filipowicz et al., 2008). Currently the bulk of research into these regulatory mechanisms has involved bioinformatics predictions in mammals (Tsang et al., 2007, Re et al., 2009),

and to date not many of these predictions have been proved experimentally (Bisognin et al., 2012).

The demonstration that feed-forward regulation of *SATB1* by FOXP3 and FOXP3-regulated miRs occurs in normal breast epithelia cells as well as in breast cancer cell lines indicated that this regulation was unlikely to be an artefact of *FOXP3* overexpression. Future studies comparing FOXP3, miR-7 and miR-155 levels with SATB1 protein levels in matched human normal and disease breast tissue samples will be important for establishing if there is a correlation between their expression levels in primary tumour samples. These tumour samples would need to be categorised based on tumour origin, stage, metastatic status and ER/PR/HER-2 status to allow for clearer definition of the roles these genes play in cancer. In addition, to strengthen the link between FOXP3-mediated regulation of *SATB1* with a reduction in metastatic potential, it would also be of interest to test the metastatic and growth activities of breast cancer cell lines in which *FOXP3*, miR and *SATB1* levels have been manipulated in *in vivo* murine models of metastasis. For example, mouse xenograft models in which MDA-MB-231 cells are injected directly into the mammary fat pad of immunocompromised SCID mice is a well established model for tumour progression, with the formation of primary tumours and subsequent development of metastases resembling the multiple stages involved in malignant breast cancer in human patients (Fantozzi and Christofori, 2006, Kochetkova et al., 2009). It would also allow uncoupling of transcription factor regulation from miR regulation, thus providing further insight into the roles of the individual regulatory components.

Although there has recently been increased interest in the role of SATB1 in cancers, there is still little known about how the gene is regulated or how it is induced in the transition

from normal to cancerous breast epithelium. In particular, the mechanism by which *SATB1* expression is upregulated in cancer is unknown. It is possible that this upregulation is a result of activation of a normally silent gene- although this is most commonly associated with the creation of fusion genes (Edwards, 2010). The other possibility is that upregulation results from aberrant expression of a gene that is normally under tight control; depending on the developmental and differential state of the cell (Savarese et al., 2009). In other cell types, *SATB1* and the highly related family member *SATB2* are involved in stem cell self-renewal and differentiation decisions in trophoblast and embryonic stem cells (Asanoma et al., 2012, Savarese et al., 2009), with high expression of the *SATB* genes associated with the stem cell state, with reduced expression observed following a decrease in cell renewal and increased differentiation. In addition, SATB1 has been shown to play an important role in the activation of differentiation programs in epidermal progenitor cells (Fessing et al., 2011) and naive T cells (Alvarez et al., 2000). Therefore it is possible that low level *SATB1* expression is required in normal breast epithelial stem cells (MaSC) or multipotent progenitor cells, with *SATB1* overexpression in epithelial cells a possible consequence of the reactivation or gain of 'stem-like' characteristics. Consistent with this proposal, more aggressive breast cancer cells such as those that overexpress *SATB1*, have been proposed to contain higher proportions of cancer stem cells (Visvader and Lindeman, 2008, Shipitsin et al., 2007) and also to have lower levels of miR-7 when compared with less aggressive cell lines (Wang et al., 2012).

As well as describing a role for FOXP3 and FOXP3-regulated miRs in the regulation of *SATB1*, the work in this thesis linked FOXP3, via upregulation or maintenance of high levels of miR-7, to reduced proliferative activity, invasive ability and resistance to apoptosis. Some of these effects could be due to changes in *SATB1* as its expression has

been linked to changes in the expression of proliferation associated genes in epidermal cells (Fessing et al., 2011) and breast cells (Han et al., 2008). The demonstration that FOXP3 positively regulates miR-7, a microRNA previously linked to the regulation of growth factor (GF) receptor signalling pathways, including the EGFR and IGFR pathways, links *FOXP3* expression to these pathways. Interestingly, previous studies have also reported that FOXP3 suppresses the transcription of *HER2*, a second member of the EGFR family, in breast epithelia (Zuo et al., 2007b), and that increased *SATB1* expression in aggressive breast cancer results in the upregulation of *HER2* (Han et al., 2008). These findings suggest that one of the key functions of FOXP3 in breast epithelial cells is to limit the response to growth factors such as EGF by repressing the levels of growth factor receptors and intracellular signalling molecules.

The EGFR/ERb-B family of tyrosine kinases each have important roles in normal breast development, including cell proliferation and differentiation (Eccles, 2011). These receptors (EGFR, HER2, HER3 and HER4) are able to form homodimers and heterodimers in response to ligand binding, allowing the formation of different dimer pairs that generates additional flexibility to drive a number of cellular responses (Eccles, 2011). Recent studies have found evidence to support a role for the EGFR signalling pathway in the control of myoepithelial cell fate in human mammary tissues, with EGF levels determining if cells undergo proliferation or differentiation (Pasic et al., 2011). This suggests a role for miR-7 in the breast, as high levels of EGFR signalling result in proliferation, while low levels result in differentiation. Components of the EGFR signalling pathway are often overexpressed in cancers (Kefas et al., 2008, Webster et al., 2009), where they are associated with disease progression, poor prognosis and resistance to therapy (Bianco et al., 2005). The current clinical responses of tumours to existing anti-EGFR treatments,

such as cetuximab and erlotinib, are limited and have a high failure rate, so there is a need to establish new approaches for blocking EGFR signalling (Webster et al., 2009, Bianco et al., 2005, Bertucci et al., 2012). Published data have implicated a role for miR-7 in the regulation of EGFR signalling, through the suppression of pathway members including *EGFR*, *RAF-1* and *PAK-1* (Reddy et al., 2008, Fang et al., 2012, Webster et al., 2009), consistent with results of this thesis. Other established targets of miR-7 include the Insulin receptor substrates (*IRS*) IRS-1 and IRS-2, which are regulators of the proliferation driver serine/threonine protein kinase AKT (Reddy et al., 2008) and insulin growth factor receptor 1 (IGFR-1), which has been shown to promote metastasis in cancers (Zhao et al., 2012). MiR-7 can regulate a broad range of targets, unlike current inhibitors that target a single molecule (such as antibodies and TK inhibitors), and therefore there is a reduced risk that cells will develop a resistance. Also, re-introducing tumour suppressor function is theoretically better than reducing oncogene function, as this is more likely to be well tolerated by normal tissues. Indeed, treatment of mouse models with miR therapies has been shown to be both safe and effective (Gandellini et al., 2011, Bader et al., 2010). However there are also potential drawbacks, as an effective method for targeted delivery would need to be established, and in the case of miR-7, its different role in ER^+ vs. ER^- cancers needs to be addressed.

Preliminary studies examining the usefulness of miR-7 as a therapy to suppress the EGFR signalling pathway in other cancers has begun. For example, Rai *et al.* have demonstrated that delivery of plasmids expressing miR-7 by cationic liposomes into lung cancer cells *in vivo* has therapeutic potential for overcoming resistance to EGFR tyrosine kinase inhibitor treatments (Rai et al., 2011). Injection of the miR-7 plasmids not only revealed significant tumour regression in mouse lung cancer models, but also resulted in reduced expression of

EGFR, *RAF-1* and *IRS-1* (Rai et al., 2011). This indicates the potential benefit of miR-7 as a therapeutic agent for the treatment of lung cancer, and it would therefore be of interest to perform similar experiments in mouse models of breast cancer. In breast epithelia, the transcription of miR-7 has now been linked to the tumour suppressors HoxD10 (Reddy et al., 2008) and FOXP3 (this thesis) and therefore restoration of this transcriptional control by increasing the expression of these factors could potentially be an alternative therapeutic. Preliminary evidence suggests that this is possible, as agents that activate ATF2/c-Jun can upregulate FOXP3 and miR-7 levels in breast cancer cell lines, however this should be treated with caution, as results in this thesis suggest that agents such as Anisomycin may be toxic to cells.

Overall, this thesis proposes that FOXP3 plays an important tumour suppressive role in breast epithelial cells, with expression of this transcription factor required for the maintenance of steady-state miR-7 and miR-155 levels necessary for normal breast epithelial cell homeostasis. This work provides the first evidence to suggest that expression of *FOXP3* in breast cancer cells will result in FOXP3-mediated upregulation of miR-7 and miR-155, leading to the downregulation of the pro-metastatic oncogene *SATB1* and members of the EGFR pathway. This important insight into the progression of cancer suggests that altering expression of *FOXP3* or its downstream targets could prove to be effective breast cancer therapies, with such treatment potentially resulting in restoration of the FOXP3-mediated homeostasis seen in healthy breast epithelial cells or a reduction in breast cancer severity. Of note, this thesis has focused on the regulation of miR-7, miR-155 and *SATB1* as targets of FOXP3-mediated repression. It is extremely likely based on previous studies in Treg cells, that FOXP3 regulates a number of other targets in breast

epithelia, including other microRNAs and transcription factors that may be involved in its tumour suppressive activity.

APPENDIX

7.1 Supplementary Data

Figure 5.S1 *Both isoforms of FOXP3 are present in the HMEC cell line*

a)

Homo sapiens forkhead box P3 (FOXP3), transcript variant 1, mRNA
Length=2292
GENE ID: 50943 FOXP3 | forkhead box P3 [Homo sapiens] (Over 100 PubMed links)
Score = 1796 bits (972), Expect = 0.0
Identities = 1004/1007 (99%), Gaps = 0/1007 (0%)
Strand=Plus/Plus

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Sbjct  106   TGATACGTGACAGTTTCCCACAAGCCAGGCTGATCCTTTTCTGTCAGTCCACTTCACCAA  165

Query  138   GCCTGCCCTTGGACAAGGACCCGATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCCTT  197
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Query  198   CCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGGAGGGCTGCACCCAAAGCCT  257
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Query  258   CAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGGCG  317
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Sbjct  466   TGATCAGCCTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCCTG  525

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Sbjct  526   GCCTCCCACCTGGGATCAACGTGGCCAGCCTGGAATGGGTGTCCAGGGAGCCGGCACTGC  585

Query  558   TCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACCCTTTCGGCTGTGCCCC  617
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Sbjct  586   TCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACCCTTTCGGCTGTGCCCC  645

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Query  738   GCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGTGC  797
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Query  798   TGGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTGGCTGGGAAAATGGCACTGACCA  857
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Query  918   AAGGCCCTGTCGTCCCAGCCTGGTCTGGCCCCCGGGAGGCCCCTGACAGCCTGTTTGCTG  976
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Sbjct  946   AAGGCCCTGTCGTCCCAGCCTGGTCTGGCCCCCGGGAGGCCCCTGACAGCCTGTTTGCTG  1005

Query  977   TCCGGAGGCACCTGTGGGGTANCCATGGAAACAGCACATTCCCAGA  1022
              |||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  1006  TCCGGAGGCACCTGTGGGGTAGCCATGGAAACAGCACATTCCCAGA  1050
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b)

Homo sapiens forkhead box P3 (FOXP3), transcript variant 2, mRNA
Length=2292

GENE ID: 50943 FOXP3 | forkhead box P3 [Homo sapiens] (Over 100 PubMed links)

Score = 1707 bits (924), Expect = 0.0
Identities = 938/947 (99%), Gaps = 3/947 (0%)
Strand=Plus/Plus

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Query  76   TTTGATACGTGACAGTTTCCCACAAGCCAGGCTGATCCTTTTCTGTCAGTCCACTTCACC  135
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Sbjct  104  TTTGATACGTGACAGTTTCCCACAAGCCAGGCTGATCCTTTTCTGTCAGTCCACTTCACC  163

Query  136  AAGCCTGCCCTTGGACAAGGACCCGATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCC  195
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Sbjct  164  AAGCCTGCCCTTGGACAAGGACCCGATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCC  223

Query  196  TTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGGAGGGCTGCACCCAAAGC  255
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Sbjct  224  TTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGGAGGGCTGCACCCAAAGC  283

Query  256  CTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGG  315
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Sbjct  284  CTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGG  343

Query  316  CGGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTCTC  375
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  CGGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTCTC  403

Query  376  AACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAGC  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  AACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAGC  463

Query  436  CATGATCAGCCTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCC  495
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464  CATGATCAGCCTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCC  523

Query  496  TGGCCTCCCACCTGGGATCAACGTGGCCAGCCTGGAATGGGTGTCCAGGGAGCCGGCACT  555
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524  TGGCCTCCCACCTGGGATCAACGTGGCCAGCCTGGAATGGGTGTCCAGGGAGCCGGCACT  583

Query  556  GCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACCCTTTCGGCTGTGCC  615
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584  GCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACCCTTTCGGCTGTGCC  643

Query  616  CCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGGT  675
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  644  CCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGGT  703

Query  676  CTTCGAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGAA  735
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704  CTTCGAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGAA  763

Query  736  GGGCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGT  795
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764  GGGCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGT  823

Query  796  GCTGGAGAAGGAGAAGCTGAGTGCCATGCANGCCCACCTGGCTGGGAAAATGGCACTGAC  855
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  824  GCTGGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTGGCTGGGAAAATGGCACTGAC  883

Query  856  CAAGGCTTCATCTGTGGCATCATCCGACAAGGGCTCCTGCTGCATCGTAGCTGCTGGCAG  915
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  884  CAAGGCTTCATCTGTGGCATCATCCGACAAGGGCTCCTGCTGCATCGTAGCTGCTGGCAG  943

Query  916  CCANGNCCCTGTCGTCCCAGCCCTGGTCTGGCCCNNGGGAGGCCCCT  960
            ||| | ||||||||||||||| |||||||||||  ||||||||||||
Sbjct  944  CCAAGGCCCTGTCGTCCCAGCC-TGGTCTGGCCCCCGGGAGGCCCCT  989
```

BLAST analysis of sequencing results identified both the full length (a) and Δ3 (b) isoforms of *FOXP3* in HMECs. Total HMEC RNA was isolated, converted to cDNA and then *FOXP3* amplified by PCR before Big Dye sequencing reactions were performed (as described in Section 2.2.15).

Figure 5.S2 ***Only the Δ3 isoform of FOXP3 is present in the MCF10a cell line***

```
      Homo sapiens forkhead box P3 (FOXP3), transcript variant 2, mRNA
                                Length=2292
GENE ID: 50943 FOXP3 | forkhead box P3 [Homo sapiens] (Over 100 PubMed links)
                  Score = 1687 bits (913),  Expect = 0.0
              Identities = 925/933 (99%), Gaps = 2/933 (0%)
                        Strand=Plus/Plus

 Query  18   GGTCTGCGGCTTCCACACCGTACAGCGTGGTTTTTCTTCTCGGTATAAAAGCAAAGTTGT  77
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  43   GGTCTGCGGCTTCCACACCGTACAGCGTGGTTTTTCTTCTCGGTATAAAAGCAAAGTTGT  102

Query  78   TTTTGATACGTGACAGTTTCCCACAAGCCAGGCTGATCCTTTTCTGTCAGTCCACTTCAC  137
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103  TTTTGATACGTGACAGTTTCCCACAAGCCAGGCTGATCCTTTTCTGTCAGTCCACTTCAC  162

Query  138  CAAGCCTGCCCTTGGACAAGGACCCGATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCC  197
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  163  CAAGCCTGCCCTTGGACAAGGACCCGATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCC  222

Query  198  CTTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGGAGGGCTGCACCCAAAG  257
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223  CTTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGGAGGGCTGCACCCAAAG  282

Query  258  CCTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAG  317
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283  CCTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAG  342

Query  318  GCGGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTCT  377
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343  GCGGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTCT  402

Query  378  CAACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAG  437
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  403  CAACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAG  462

Query  438  CCATGATCAGCCTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGC  497
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  463  CCATGATCAGCCTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGC  522

Query  498  CTGGCCTCCCACCTGGGATCAACGTGGCCAGCCTGGAATGGGTGTCCAGGGAGCCGGCAC  557
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  523  CTGGCCTCCCACCTGGGATCAACGTGGCCAGCCTGGAATGGGTGTCCAGGGAGCCGGCAC  582

Query  558  TGCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGTACCCTTTCGGCTGTGC  617
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  583  TGCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACCCTTTCGGCTGTGC  642

Query  618  CCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGG  677
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  643  CCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGG  702

Query  678  TCTTCGAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGA  737
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  703  TCTTCGAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGA  762

Query  738  AGGGCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGG  797
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  763  AGGGCAGGGCACAATGTCTCCTCCAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGG  822

Query  798  TGCTGGAGAAGGAGAAGCTGAGTGCCATGCANGCCCACCTGGCTGGGAAAATGGCACTGA  857
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  823  TGCTGGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTGGCTGGGAAAATGGCACTGA  882

Query  858  CCAAGGCTTCATCTGTGGCATCATCCGACAAGGGNNTCCTGCTGCATCGTAGCTGCTGGC  917
             ||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||
Sbjct  883  CCAAGGCTTCATCTGTGGCATCATCCGACAAGGGC-TCCTGCTGCATCGTAGCTGCTGGC  941

             Query  918  AGCCANNGCCCTGTCGTCCCAGNCTGGGTCTGG  950
                    |||||  ||||||||||||||| |||| |||||
             Sbjct  942  AGCCAAGGCCCTGTCGTCCCAGCCTGG-TCTGG  973
```

BLAST analysis of sequencing results only identified the Δ3 isoform of *FOXP3* in MCF10a cells. Total MCF10a RNA was isolated, converted to cDNA and then *FOXP3* amplified by PCR before Big Dye sequencing reactions were performed (as described in Section 2.2.15).

7.2 Published Work

FOXP3 AND FOXP3-REGULATED MICRORNAS SUPPRESS SATB1 IN BREAST CANCER CELLS

McInnes N[1,2]**,** Sadlon TJ[1], Brown CY[1], Pederson S[1,2], Beyer M[3], Schultze JL[3], McColl S[4], Goodall GJ[5,6], Barry SC[1,2,7]

[1]Women's and Children's Health Research Institute, Molecular Immunology Laboratory, Women's and Children's Hospital, North Adelaide, South Australia, Australia; [2]Department of Paediatrics, The University of Adelaide, Adelaide, South Australia, Australia; [3]LIMES-Institute, Laboratory for genomics and Immunoregulation, University of Bonn, Bonn, Germany; [4]Department of Immunology, The University of Adelaide, Adelaide, South Australia, Australia; [5]Centre for Cancer Biology, SA Pathology, Adelaide, South Australia, Australia; [6]Department of Medicine, University of Adelaide, Adelaide, South Australia, Australia; [7]Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, South Australia, Australia.

Oncogene 2012; February: 1045-1054

7.2.1 Statement of Authorship

"FOXP3 and FOXP3-regulated microRNAs suppress SATB1 in breast cancer cells"

Oncogene- Feb 2012 23;31(8): 1045-1054

Natasha J McInnes (Candidate)

Performed all experiments, performed analysis of results, interpreted data, and co-wrote the manuscript.

I hereby certify that the statement of contribution is accurate.

Signed..... Date.....

Timothy J Sadlon

Supervised development of work, involved in experimental design, assisted with data interpretation, co-wrote the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... Date.....

Simon C Barry

Supervised development of work, involved in experimental design, assisted with data interpretation, co-wrote the manuscript and acted as corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... Date.....

Cheryl Y Brown

Contributed intellectually and assisted with experimental technique.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... *Date*.....

Stephen M Pederson

Performed statistical analysis of data.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... *Date*.....

Shaun McColl

Contributed intellectually and supervised work.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed .... *Date*... 18/8/2017

Gregory J Goodall

Contributed intellectually, supplied reagents and assisted with experimental technique.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... *Date*...8 August 2012...

Marc Beyer

Supplied reagents and assisted with experimental technique.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... *Date*.....16/08/12

Joachim L Schultze

Supplied reagents and assisted with experimental technique.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed..... *Date*.....10/08/12

McInnes, N**.,** Sadlon, T.J., Brown, C.Y., Pederson, S., Beyer, M., Schultze, J.L., McColl, S., Goodall, G.J., Barry, S.C. (2012)
FOXP3 AND FOXP3-Regulated MicroRNAs suppress SATB1 in breast cancer cells.
Oncogene, v. 31, pp. 1045-1054

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